Novel enzymes from the radioresistant bacterium *Deinococcus radiodurans* with potential roles in DNA repair

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2 ABSTRACT

Deinococcus radiodurans is a bacterium showing an amazing resistance to ionising radiation. Actively growing cultures show no detectable loss of viability up to 5,000 Gray, which corresponds to approximately 1,600 double-strand breaks per cell. DNA double-strand breaks are the most lethal kind of DNA damage and few double-strand breaks are usually enough to kill a cell. Although the *Deinococcus radiodurans* genome has been sequenced, it remains unclear which factors contribute to the extremely resistant phenotype. It has been suggested, that the enhanced radioresistance results from an optimal combination of various active and passive mechanisms present in many other radiosensitive organisms. These might include:

(i) a potent machinery to deal with oxidative stress,
(ii) a robust cell wall,
(iii) high levels of Mn(II) to protect proteins and DNA,
(iv) mechanisms to prevent degradation of free DNA ends,
(v) an ordered structure of the DNA,
(vi) genome multiplicity,
(vii) a sophisticated regulation of DNA repair, transcription and cell cycle progression and
(viii) multiple DNA repair pathways with efficient and specialised enzymes.

The aim of this thesis was to identify and characterise novel DNA repair enzymes that are likely to contribute to the extremely high DNA repair capacity of this bacterium. It seems that *Deinococcus radiodurans* possesses a rather advanced DNA repair apparatus compared to other well-characterised bacteria such as *Escherichia coli*. Several genes of *Deinococcus radiodurans* seem to originate from eukaryotes and might have been obtained via horizontal gene transfer. This work focuses on five proteins that are expected to at least partially account for DNA double-strand break repair. In the first part of this work an enzyme has been characterised that is a member of the family X of DNA polymerases. Surprisingly, this protein contains a structure-modulated nuclease activity, suggesting a role in resolving DNA structures that would otherwise prevent further processing by DNA repair enzymes. Deletion of the gene leads to a radiosensitive phenotype with a delay in double-strand break repair. The second part focuses on DNA ligation and end-healing in *Deinococcus radiodurans.* Characterisation of an NAD^+-dependent DNA ligase showed a high ligation activity in the presence of Mn(II) and NAD^+. A second ATP-dependent DNA ligase has been predicted by sequence alignments. This enzyme indeed might play a role in DNA repair, as it is part of an operon that is highly upregulated upon irradiation. The recombinant protein possesses adenylyltransferase activity in the presence of ATP and is specifically adenylated on lysine

40. However, no DNA ligation activity could be detected under any conditions tested. The second protein of the same putative DNA repair operon turned out to be a polynucleotide kinase that can heal modified DNA termini and prepare them for religation. Mutation of arginine 371 strongly reduces the polynucleotide kinase activity. The third protein of this putative DNA repair operon interestingly shows some sequence similarity to the family of Macro domains. The members of this family are known to bind ADP-ribose monomers and polymers. Indeed a specific binding to ADP-ribose could be detected with this protein. Further work is required to test whether ADP-ribosylation plays a role in bacterial DNA repair and might be involved in signalling, regulation or interactions with other proteins.

3 ZUSAMMENFASSUNG

Deinococcus radiodurans ist ein Bakterium mit einer sehr hohen Resistenz gegenüber ionisierender Strahlung. Aktiv wachsende Kulturen zeigen keinen messbaren Verlust ihrer Lebensfähigkeit bis zu einer Dosis von 5000 Gray, was ungefähr 1600 Doppelstrangbrüchen in der Desoxyribonukleinsäure (DNS) entspricht. DNS-Doppelstrangbrüche sind die gefährlichsten aller DNA Schäden, und normalerweise sind wenige Doppelstrangbrüche ausreichend, um eine Zelle zu töten. Obwohl das Genom von *Deinococcus radiodurans* sequenziert ist, weiss man bisher nicht, welche Faktoren zu dieser extremen Radioresistenz beitragen. Es wird angenommen, dass diese auf einer optimalen Kombination verschiedener aktiver und passiver Mechanismen basiert, die auch von anderen nicht radioresistenten Organismen bekannt sind. Dazu gehören vermutlich:

(i) eine potente Maschinerie, um den oxidativen Stress zu bewältigen,
(ii) eine robuste Zellwand,
(iii) ein hoher Gehalt an Mn(II) zum Schutz von Proteinen und DNS,
(iv) Mechanismen, die einen Abbau der freien DNS-Enden verhindern,
(v) eine geordnete DNS-Struktur,
(vi) mehrere Genomkopien,
(vii) eine differenzierte Regulation der DNS-Reparatur, der Transkription und des Zellzyklus sowie
(viii) diverse Wege zur DNS-Reparatur mit effizienten und spezialisierten Enzymen.

Das Ziel dieser Doktorarbeit war die Identifizierung und Charakterisierung neuer DNS-Reparaturenzyme, die wahrscheinlich einen Beitrag leisten können zur extrem hohen DNS-Reparaturkapazität von *Deinococcus radiodurans*. Anscheinend hat dieses Bakterium einen erweiterten DNS-Reparaturapparat im Vergleich zu anderen gutuntersuchten Bakterien wie beispielsweise *Escherichia coli*. Mehrere Gene von *Deinococcus radiodurans* scheinen von Eukaryoten abzustammen und wurden vermutlich über horizontalen Gentransfer übertragen. Fünf Proteine, für die eine Beteiligung an der Doppelstrangbruchreparatur angenommen wird, wurden im Rahmen dieser Dissertation genauer untersucht. Das erste untersuchte Enzym ist ein Mitglied der DNS-Polymerase-Familie X. Überraschenderweise zeigt dieses Enzym eine strukturmodulierte Nukleaseaktivität, was eine Aufgabe bei der Auflösung von DNS-Strukturen nahelegt, die sonst nicht weiter prozessiert werden könnten. Wird das entsprechende Gen entfernt, zeigen die Bakterien einen radiosensitiven Phänotyp und eine verzögerte Doppelstrangbruchreparatur.

Im zweiten Teil der Arbeit wurde die DNS-Ligation und die Heilung von modifizierten DNS-Enden untersucht. Die Charakterisierung einer NAD^+-abhängigen DNS-Ligase zeigt eine hohe Ligationsaktivität in Gegenwart von NAD^+ und Mn(II). Aufgrund von Sequenzvergleichen wurde eine zweite ATP-abhängige DNS-Ligase vorhergesagt. Dieses Enzym könnte in der Tat eine Rolle bei der DNS-Reparatur spielen, da es zu einem Operon gehört, welches durch Bestrahlung stark induziert wird. Das rekombinante Protein zeigt Adenylyltransferase-Aktivität mit ATP und wird spezifisch am Lysin 40 adenyliert. Allerdings konnte unter keinen Bedingungen DNS-Ligation beobachtet werden. Ein weiteres Protein desselben Operons erwies sich als eine Polynukleotidkinase, welche modifizierte DNS-Enden heilen und für die Ligation vorbereiten kann. Mutation von Arginin 371 reduzierte die Polynukleotidkinaseaktivität. Interessanterweise zeigt das dritte Protein des mutmasslichen Reparaturoperons eine gewisse Ähnlichkeit zur Familie der Macrodomänen, deren Mitglieder ADP-Ribose als Monomer oder Polymer binden können. In der Tat stellte sich heraus, dass dieses dritte Operonprotein ADP-Ribose binden kann. Weitere Experimente sind erforderlich, um zu erfahren, ob ADP-Ribosylierung bei der bakteriellen DNS Reparatur eine Rolle spielen und zum Beispiel an der Signalübertragung, Regulation oder an Interaktionen mit anderen Proteinen beteiligt sein könnte.

4 ABBREVIATIONS

aa	amino acid
Amp	ampicillin
AMP	adenosine 5´-monophosphate
ATP	adenosine 5´-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
B. subtilis	*Bacillus subtilis*
°C	degree Celsius
Ci	curie
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
D. radiodurans	*Deinococcus radiodurans*
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra acetate
EMSA	electrophoretic mobility shift assay
FPLC	fast pressure liquid chromatography
g	acceleration due to gravity
g	gram
His	histidine
IPTG	isopropyl-β-D- thiogalactopyranoside
kb	kilo base(s)
kDa	kilo Dalton
LB medium	Luria- Bertani medium
LMW marker	low molecular weight standard markers
M	molar (concentration)
2-ME	2-mercaptoethanol
mg	milligram(s)
µg	microgram(s)
min	minute(s)
mol	mole(s)
µmol	micromole(s)
M_r	relative molecular mass
NAD^+	Nicotinamide adenine dinucleotide, oxidised
ng	nanogram(s)
nm	nanometer
$OD_{(600)}$	optical density $_{(wavelength)}$
o/n	overnight (at least 14 hours of incubation)
PCR	polymerase chain reaction
pmol	picomole(s)
PMSF	phenyl- methylsulfonyl fluoride (serine -protease inhibitor)
pol	DNA polymerase
rpm	rounds per minute
RNA	ribonucleic acid
RT	room temperature
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ss	single stranded
Tris	Tris-(hydroxymethyl)-aminoethane
U	unit [µmol/min]
UV	ultra violet
(v/v)	volume per volume
wt	wild-type

5 INTRODUCTION

5.1. *Deinococcus radiodurans* - a model organism to study DNA repair

Deinococcus radiodurans (*D. radiodurans*) is a bacterium belonging to the phylum *Thermus/ Deinococcus*. *D. radiodurans* is famous for its extreme resistance to a variety of stresses, mainly for its amazing resistance to γ-radiation (Figure 1). *D. radiodurans* has been discovered in 1956 in irradiated cans of ground meat that were supposed to be sterilised. Since then, more than 20 other species have been assigned to the family of *Deinococcaceae*. Most of them have been isolated from very hostile environments such as deserts or the Antarctic, and all species described so far have been obtained from heavily irradiated samples. *D. radiodurans* is by far the best-characterised member of this family and many scientists use this model organism, especially since its genome sequence has been published in 1999 [1]. The genome consists of two chromosomes of 2.64 mega-basepairs and 0.41 mega-basepairs, a megaplasmid of 0.18 mega-basepairs and a plasmid of 0.045 mega-basepairs encoding together for more than 3,000 proteins [2].

D. radiodurans cells are pigmented and appear pink-orange. The pigmentation is required for its extreme UV-resistance. The cells normally live either as diplococci or tetracocci and contain several genome copies per cell (Figure 2). There is no consensus whether the 4 compartments of a *D. radiodurans* tetracoccus are fully separated or whether there is some communication and exchange taking place [3]. The fact that *D. radiodurans* normally does not grow as single cells might also distort some of the experimental data about survival, given that each coccus by itself could give rise to a new colony [4]. *D. radiodurans* cells stain gram-positive, but the cell wall differs from other gram-positive bacteria in respect to first its lipid composition and second by the types of layers. *D. radiodurans* grows optimal at 30°C in rich medium and is an obligate aerobe. Cells are naturally competent and many genetic tools are available to modify the bacterium for special purposes.

D. radiodurans is a suitable model organism to study mechanisms of radioresistance and in particular double-strand break repair. Knowledge about radioresistance is of great importance for several reasons: as DNA repair pathways are highly conserved throughout evolution a better understanding of these processes might as well be applicable to other organisms and reveal new evolutionary events and general principles. The growing evidence for the presence of complex DNA repair pathways in prokaryotes such as non-homologous end-joining, makes bacteria interesting objects to study such complex processes. A lot of basic knowledge is still required to understand induced radioresistance, especially the radioresistance of cancer cells, to improve radiotherapy. Further understanding of bacterial DNA repair mechanisms can also help on comprehending DNA repair defects that could lead

to cancer in humans. In addition, detailed knowledge of differences between pathogenic bacteria and other organisms can facilitate the search for new antibacterial drugs.

Besides basic research in order to gain further insight in DNA repair mechanisms, *D. radiodurans* strains are also selected and optimised for bioremediation [5]. Many radioactive mixed waste sites remained from the cold war and were disposed directly in the ground. *D. radiodurans* can be easily manipulated to degrade a variety of organic toxins and is able to survive under chronic radiation as high as 60 Grays per hour. During the last years a lot of research has been done to understand the underlying mechanisms of radioresistance and a lot of excited discussions have been going on, part of which are summarised in a review in chapter 5.4 of this work.

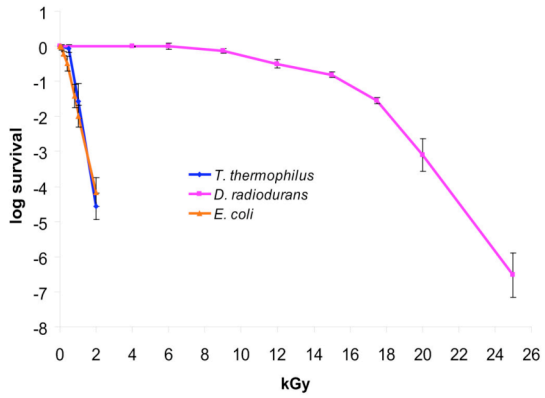

Figure 1
Radiation resistance of *T. thermophilus* (ATCCBAA-163), *D. radiodurans* (ATCCBAA-816) and *E.coli* (K12 MG1655) as measured by ^{60}Co irradiation. From Omelchenko *et al.* (2005) Comparative genomics of *Thermus thermophilus* and *Deinococcus radiodurans*: divergent routes of adaptation to thermophily and radiation resistance. BMC Evolutionary Biology 5(57), online version

5.2. Factors that might contribute to radioresistance

Ionising radiation leads to the formation of multiple ions and electrons. Reactions with other molecules in a cell then produce free radicals, which in turn can harm and modify all molecules of a cell. In living cells, reaction with water molecules forms highly reactive hydroxyl radicals. Ionising radiation is usually very toxic at low doses by generating a variety of DNA damages such as DNA strand-breaks and a broad spectrum of oxidative damages. Radiation resistant organism are therefore expected to be able to deal efficiently with DNA strand-breaks and to possess means by which formation of free radicals can be reduced or by which toxic radicals can be scavenged before the DNA molecules are damages.

The cell envelope of *D. radiodurans* has a rather unusual composition and structure and might pose a first barrier to exogenous stresses. At least 6 layers have been identified, but not all lipid components have been described so far and some seem to be unique to *Deinococcaceae* [6]. Additionally to the uncommon lipid composition a variety of proteins and carotenoids play a role for the integrity of the cell envelope and the resistance to oxidative

stress [7, 8]. Comparative genetics furthermore revealed an expanded family of membrane-associated proteins that might also play a role in cell membrane stability [9]. Recent data suggest an important role of carotenoids in stress resistance. Mutation of the phytoene synthase leads to cells without pigmentation that are sensitive to desiccation, oxidative stress and γ-irradiation [10]. Deinoxanthin, a major product of the carotenoid synthesis pathway shows an especially strong ability to scavenge H_2O_2 and single oxygen thus exhibiting a protective effect on DNA [11]. Moreover, *D. radiodurans* encodes an orphan protein, which might be involved in pigmentation. It is related to the royal jelly proteins in honeybees and to the *Drosophila melanogaster* yellow-B protein, which is known to be involved in pigmentation and development [12].

Although this had been proposed, no correlation seems to exist between the number of genome copies and the degree of radioresistance [13], but it is likely that two genome copies or more are a necessary, but not a sufficient condition for radioresistance. The same might be the case for other factors that seemed to be a major cause of radioresistance at first sight, such as a highly ordered DNA structure (see also Review in section 5.4). It was shown that the degree of DNA strand-breaks caused *in vivo* does not differ between *E. coli* and *D. radiodurans* cells, making an important role of DNA protection during irradiation rather unlikely. It is therefore assumed that *D. radiodurans* possesses more efficient ways of repairing its DNA and maybe protecting its DNA repair enzymes.

In addition, proteins that bind free DNA ends can protect them from nuclease degradation and thus prevent the loss of genetic information. The protein DdrA for instance has been shown to bind DNA ends and to play an important role for radioresistance [14, 15]. Other proteins that protect DNA ends or RNA might still be among the many orphan genes.

The genome of *D. radiodurans* contains several mobile genetic elements, but it is unclear whether they are involved in genome instability. The genome contains two unrelated prophages and has more small non-coding repeats than the *E. coli* genome. Furthermore, the *D. radiodurans* genome contains much more insertion sequence (IS) elements than all other sequenced bacterial genomes and a role of the genetic elements in recombinational repair can therefore not be excluded [6].

D. radiodurans is able to deal with high oxidative stress and several gene families involved in the response to oxidative stress are expanded. *D. radiodurans* encodes for example 3 superoxide dismutases and 3 predicted catalases [9]. High intracellular levels of Mn(II) seem to be important for *D. radiodurans* to deal with reactive oxygen species and for protection of its proteins (see also 5.4).

In contrast to *Thermus Thermophilus*, a thermophilic bacterium belonging to the same clade, *D. radiodurans* has lost 4 enzymes of the NAD^+-biosynthesis pathway and depends on exogenous sources of NAD^+ and nicotinic acid [9, 16]. *D. radiodurans* lacks genes for synthesis of lysine, methionine, branched and aromatic amino acids, although they can probably be produced from other amino acids [16]. However, a nutrient medium is required for proper recovery after irradiation, suggesting that energy consuming synthesis pathways have to be avoided [4]. Several interesting protein families are expanded in *D. radiodurans* such as transcription factors or the NUDIX hydrolase family [2, 6, 17], which contains several members that participate in radioresistance by hydrolysing damaged nucleotides. The role of DNA repair- especially the repair of DNA strand-breaks- will be further introduced and elucidated in sections 5.3, 5.4, 5.5, and 6.

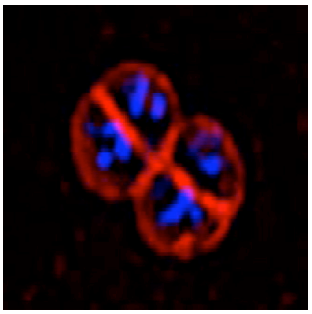

Figure 2
D. radiodurans R1 tetracoccus

From: Synopsis to Harris *et al.* (2004) DNA Damage Response Protein Buys Time for Bacterial DNA Repair (2004), PLOS Biol 2(10):e325

5.3. DNA repair in bacteria and *D. radiodurans* in particular

DNA double-strand breaks are repaired either by homologous recombination or by non-homologous end-joining. Homologous recombination uses an intact homologous DNA molecule to restore the correct DNA sequence at the site of the damage. It is the main way to repair double-strand breaks in bacteria [18]. In a first step the RecBCD complex produces a single-stranded DNA overhang and loads the RecA protein. The single-stranded DNA invades a homologous DNA molecule and new stretches of DNA are synthesised by Pol I using the intact DNA strand as a template. The remaining nicks are then sealed by DNA ligase and branch migration can occur before the so-called Holliday junctions are resolved. A novel mechanism called “extended synthesis-dependent strand annealing” has been described recently in *D. radiodurans* and will be explained in section 5.4.

Non-homologous end-joining on the other hand does not require another copy of the DNA strand [19]. It is the major pathway of double-strand break repair in eukaryotes and has only very recently been identified and characterised in bacteria [20]. In eukaryotes the DNA ends

are first recognised and bound by the Ku heterodimer. Next, proteins involved in end-processing are recruited such as the Mre11 complex, the nuclease Artemis, the Pols μ and λ and the polynucleotide kinase PNKP. Finally, the DNA ligase IV/XRCC4 complex joins the two DNA ends [19]. Non-homologous end-joining also seems to provide an important function in at least some prokaryotes. Both homo- and heterodimeric Ku-proteins have been identified in several bacteria (e.g. Mycobacteria), but not for example in *E. coli* K12 or *D. radiodurans* R1 [21]. However, it cannot be excluded that other proteins such as PprA from *D. radiodurans* [22] or Gam proteins present in some bacteria that contain Mu-like prophages [23] might have Ku-like functions. Although the genome of *D. radiodurans* contains a Mu-like prophage, no Gam-homolog has been identified [24], making the latter possibility rather unlikely for *D. radiodurans*.

Various bacteria encode more than one DNA ligase [25]. In addition to the standard bacterial NAD^+-dependent DNA ligase, sometimes an ATP-dependent DNA ligase can be found- often in an operon with a Ku homolog. Several bacterial ATP-dependent DNA ligases have been implicated in non-homologous end-joining. A small predicted ATP-dependent DNA ligase has also been identified in *D. radiodurans* [26] and has been further investigated in this work.

Also the presence of a PolX domain in some bacteria has been implicated in double-strand break repair and a PolX domain is as well present in *D. radiodurans* [27]. However, a bacterial counterpart of XRCC4 has not been identified so far. In summary, not a lot is known about the possibilities of prokaryotic non-homologous end-joining, but quite some bacteria appear to possess multiple pathways for DNA double-strand break repair and it is not yet clear whether *D. radiodurans* in one of them.

Nevertheless, sequence comparisons and experimental studies revealed that *D. radiodurans* in general encodes a typical bacterial complement of DNA repair enzymes [1, 6] and several DNA repair genes present in *E. coli* do not even have orthologs in *D. radiodurans* . For example, *D. radiodurans* seems to lack a functional SOS response system [6] and Pols of the family Y. However, considering the high number of genes without assigned function, it remains possible that some important factors have been missed so far. Further experimental work is required to identify the functions of the many orphan genes as well as confirming predicted functions. Enzymes that seem to be highly homologous to well-studied *E.coli* enzymes could possibly turn out to possess some different characteristics. Several proteins from *D. radiodurans* should be further analysed, because they (i) are likely to play an important role in DNA repair based on sequence homologies with already characterised repair enzymes, (ii) are induced upon ionising radiation or (iii) show an interesting mutant phenotype. A list of predicted proteins that should be characterised in the future is

summarised in Table 1. One interesting protein to be mentioned is LigT (DR2339), a putative 2'-5' RNA ligase. Expression of the gene is strongly induced by ionizing radiation and deletion of the gene results in a radiosensitive phenotype [15, 26]. *D. radiodurans* encodes two additional predicted phosphodiesterases of the same family (DR1000 and DR1814) that might be involved in RNA metabolism or signal transduction [6]. Another RNA ligase (DRB0094) was found to be upregulated upon γ-irradiation [26] and has been characterised as an Rnl2-like RNA ligase [28]. Like most other enzymes from *D. radiodurans* it also relies on Mn(II) as a cofactor, but whether RNA repair is important for radioresistance or whether RNA ligation is required for immunity to toxic RNases is not yet answered.

In 2004 Tanaka *et al*. found several transcripts that are highly induced both by desiccation and ionising radiation of *D. radiodurans* cells [15]. They were called ddrA, ddrB, etc for *D*NA *d*amage *r*esponse. The ones that were most highly induced, ddrA-ddrD and another gene called pprA, seem to function in RecA-independent processes. DdrA and PprA have been implicated in DNA end binding, but the *in vivo* roles of these interesting proteins still remain to be elucidated.

The DNA gyrase of *D. radiodurans* is currently also further characterised. Preliminary data show DNA-dependent ATPase and DNA helicase activities as well as similarities to topoisomerases (I. Shevelev, personal communication). Both subunits are strongly induced upon ionising radiation and the enzyme is presumably involved in DNA repair, either directly or indirectly by facilitating a quick adaptation of the DNA structure. Of special interest is also the protein Rsr, an ortholog of the eukaryotic Ro ribonucleoprotein. Ro ribonucleoproteins are a class of antigenic ribonucleoproteins associated with rheumatic autoimmune diseases. Rsr has been shown to contribute to the high UV-resistance of *D. radiodurans* [29] and recently the crystal structure of this ribonucleoprotein has been published [30]. Eukaryotic Ro proteins are found in association with mutated or damaged RNA molecules, suggesting a role in RNA quality control. As Rsr is the first prokaryotic ortholog available it would be exciting to test whether a similar function is present for the bacterial ortholog. Other proteins listed in Table 1 include nucleases, DNA helicases and proteins of completely unknown functions. In this work some of the proteins with predicted functions are described and characterised: LigA, a predicted NAD^+-dependent DNA ligase, which is slightly downregulated upon ionising radiation and a putative DNA repair operon DRB0098-DRB0100, which is strongly induced by ionising radiation (see Table 1 and section 6.2.).

5.4 *Deinococcus radiodurans*: How to survive extremes?

(Review)

In preparation for publication, 2007

Deinococcus radiodurans: How to survive extremes?

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Abstract

Ionising radiation directly and indirectly causes a variety of lesions in the molecules of a cell. The most dangerous lesions resulting from ionising radiation are probably DNA double-strand breaks. Most living organisms can only poorly cope with more than a few of such lesions. *Deinococcus radiodurans*, one of the most radioresistant organisms known to date, is able to efficiently repair hundreds of DNA double-strand beaks and is therefore used as a model organism to study what it takes to become radioresistant.

Here we review recent discoveries concerning several aspects of resistance to ionising radiation. We discuss different factors that are likely to contribute to radioresistance when combined and properly coordinated.

Deinococcus radiodurans and its expanding family

Deinococcus radiodurans (*D. radiodurans*) is a radioresistant bacterium belonging to the family of *Deinococcaceae*, which comprises more than twenty described species up to date (Table 1). Most *Deinococcaceae* are mesophiles and live as single-cells, pairs or tetrads in liquid culture with a doubling time of 1.5-3 hours. Among these species, *D. radiodurans* is by far the best characterised and was first isolated more than fifty years ago by Anderson *et al.*, who analysed canned meat that had been irradiated at 4,000 Grays in order to achieve sterility [1]. *D. radiodurans* contains multiple genome copies per cell [2], and its genome has been sequenced in 1999 [3]. The genome consists of two chromosomes, a megaplasmid and a smaller plasmid encoding approximately 3,200 proteins. The genome of *D. geothermalis*, has also been sequenced, but both genome sequences did not provide an explanation for the resistant phenotype of the *Deinococcaceae*.

D. radiodurans can survive the extremely high dose of 5,000 Grays without loss of viability [4]. This dose causes approximately 1,600 DNA double-strand breaks per cell, in comparison only few of them can usually kill an *E. coli* cell [5]. Ionising radiation also causes a variety of

single-strand damages, such as breaks and base modifications. Double-strand breaks however, require a much more elaborate repair and are therefore most lethal.

It is generally believed that the resistance to radiation results from a selection for desiccation resistance. Several members of the *Deinococcus* family have been discovered in extremely dry habitats (Table 1) and several links between radioresistance and desiccation tolerance have been described [6-8]. During the last years several new species of the *Deinococcaceae* have been discovered and characterised. All of them have been isolated from samples that had been exposed to an immense dose of ionising radiation. Nevertheless, the possibility of a radiosensitive *Deinococcus* species remains, even though it would probably be a challenge to isolate it. Several of the current hypotheses could be tested on such a close relative of *D. radiodurans* and this would possibly provide exciting new details on radioresistance.

Recently a novel species has been isolated, which belongs to the phylum *Deinococcus/Thermus* but represents a new family, the *Trueperaceae* (Table 1). *Truepera radiovictrix* is thermophilic and extremely ionising radiation resistant, whereas all *Thermus* family members are thermophilic and radiosensitive. *D. radiodurans* is one of the most radioresistant organisms known and a constantly increasing group of scientists tries to understand the underlying mechanisms. Many genetic tools are available for *D. radiodurans* and the cells are naturally competent. Possible passive and enzymatic contributions to its radioresistance have been soundly reviewed and discussed [9], but today several aspects of radioresistance still remain controversial. In this review we summarise and discuss the main theories for *D. radiodurans* radioresistance in respect to the most recent data available.

DNA repair proteins

Two proteins have been clearly implicated in DNA repair and radioresistance of *D. radiodurans*: (i) Pol I, which has been shown to be important for resistance to UV and ionising radiation [10], and (ii) RecA, which is strongly expressed upon irradiation. RecA cannot be replaced by its *E.coli* homolog [11] as it promotes DNA strand-exchange via an inverted pathway [12]. Both proteins are involved in a recently discovered two-stage DNA repair process called extended synthesis-dependent strand-annealing [13]. For this pathway a minimum of two genome copies and randomly distributed strand breaks are required. First, Pol I uses chromosome fragments with overlapping homologies both as templates and primers for RecA-independent DNA synthesis, then RecA-mediated cross-overs and recombinations take place to reassemble an intact chromosome. It remains to be elucidated how *D. radiodurans* prevents that the initial pairing occurs at a repeated DNA sequence from

the wrong part of the genome and how the individual proteins ensure efficient and faithful repair.

Various other proteins show indications for a role in radioresistance and one should keep in mind that out of the total 3,187 predicted protein coding genes only 1,696 could be assigned to a specific role category [3, 14]. And even though *D. radiodurans* encodes a typical bacterial complement of DNA repair enzymes [3, 15] at first sight, enzymes that seem to be highly homologous to well-studied *E.coli* enzymes could possibly turn out to possess some different characteristics. This has been the case for several enzymes from *D. radiodurans* that have been analysed recently and some of which are listed in Table 2.

A group of genes that are upregulated in response to either desiccation or ionising radiation has been identified in 2004 [8]. The gene products were called DdrA, DdrB, DdrC, DdrD and PprA and deletion mutants confirmed their importance. DdrA has been further characterised and turned out to play a role in protection of free DNA ends [16], but the functions of DdrB, DdrC, and DdrD remain unclear. PprA has been shown to bind and tether free DNA ends and might be involved in DNA ligation [17, 18]. However, further research is required to show a possible physical or functional interaction with the *D. radiodurans* DNA ligase.

The *D. radiodurans* gyrase has not yet been characterised, but it seems likely that the enzyme is required for DNA restructuring and repair and both subunits are induced by γ-radiation [8, 19]. DNA repair enzymes involved in UV-resistance that have been characterised so far include the excinuclease subunit UvrA [20, 21] and the UV endonuclease beta, which uses Mn(II) as a cofactor and incises DNA by true endonuclease action [22, 23]. UvrA, UvrB, RuvA, GyrA and GyrB are all induced both by IR and desiccation, suggesting a role in DNA strand-break repair.

Several proteins seem to be involved in RecA-independent DNA repair pathways (e.g. DdrB, PprA and PolX). However, even though in *B. subtilis*, *M. tuberculosis* and other prokaryotes there is clear evidence for bacterial non-homologous end-joining systems ([24] and references therein), so far the presence of a non-homologous end-joining pathway in *D. radiodurans* has not been proven.

One protein containing a polymerase beta-like PolX domain in combination with a PHP (polymerase and histidinol phosphatase) domain has been identified in the genome of *D. radiodurans* [3]. The presence of a PolX domain is very uncommon in bacteria and archaea, but homologs have been identified in *B. subtilis* and *M. thermoautotrophicum*. Deletion of the gene leads to an increased radiosensitivity and surprisingly the enzyme shows a structure-modulated nuclease activity [25, 26]. A homolog of SbcB (exonuclease I) is absent in *D. radiodurans*, but the homologs of Rad50/SbcC and Mre11/SbcD were identified [3, 15] and

participate in DNA repair [27]. Deletion of both SbcCD and the structure-modulated nuclease PolX has an additive effect suggesting complementary roles in processing damaged DNA ends [27].

Various DNA repair enzymes turned out to possess unique domain architectures. One of them is a RecQ helicase (DR1289), which contains 3 HRD domains involved in regulation of the helicase activity (Table 2). Another putative RecQ helicase containing only one HRD domain has been identified (DR2444), but the enzymatic activity still needs to be confirmed. Another DNA helicase, the RecD protein of *D. radiodurans*, possesses an unusual domain organisation (Table 2) and exists without RecB and RecC. This is striking, because in *E.coli* the RecBCD recombinase plays a major role in the repair of DNA double-strand breaks. RecD, besides being present without RecBC, has an extended N-terminus and is involved in an antioxidant pathway [28]. *RecD* mutants are sensitive to ionising radiation, UV-light and hydrogen peroxide, but resistant to mitomycin C and methyl methane sulfonate. The mutant cells are also more easily transformable, suggesting a role in DNA repair or recombination [29].

Another interesting protein is RecR, which together with RecF and RecO helps replacing SSB by RecA and plays a role in the bacterial homologous recombination RecFOR pathway. Its eukaryotic counterpart is the protein Rad52. For *D. radiodurans* RecR a role in homologous recombination and DNA interstrand cross-link repair has been proposed [30] and the crystal structure revealed a ring-shaped tetramer that is able to open and close. Each monomer consists of an N-terminal helix-hairpin-helix motif essential for DNA binding, a C-terminal domain with a Cys4 zinc-finger motif, a Toprim domain and a Walker B motif [31]. RecO also supports DNA annealing and RecA-mediated recombination similar to eukaryotic Rad52. The crystal structure or DrRecO showed a novel alpha-helical domain, a zinc-binding domain and an OB-fold that is structurally similar to the OB-fold of ssDNA-binding proteins such as RP-A, SSB or BRCA2 [32]. The structure of *D. radiodurans* RecF, the third protein of the recombination mediator complex, has recently been solved [33]. The protein shows a strong structural similarity to the head domains of Rad50 and SMC proteins suggesting a conserved mechanism of DNA recognition and DNA binding. The protein forms dimers in the presence of ATP and a clamp-loader function for the tetrameric RecR-ring has been proposed.

Yet another protein that is involved in DNA repair and that differs from it standard bacterial homologs is SSB. *D. radiodurans.* SSB (DraSSB) is double the size of *E. coli* SSB (EcoSSB), forms dimers instead of tetramers and contains two OB-folds per monomer instead of just one [34]. The same kind of homodimer has been found in *D. murrayi* [35], but the reason for this change from a tetra- to a homodimer is not intelligible considering that DraSSB can

replace EcoSSB in *E.coli* cells. DraSSB, however, seems to have a more robust capacity to displace a short DNA strand from a DNA duplex consistent with a relevance in DNA double-strand break repair [36].

Some operons are probably related to the unique features of *D. radiodurans* and may have been required via horizontal gene transfer. One operon comprises two eukaryotic-type enzymes, a Uracil DNA glycosylase (DRB0689) and a DNA topoisomerase IB (DRB0690, see also Table 2). Another interesting operon contains a putative DNA ligase (DRB0100), a predicted polynucleotide kinase (DRB0098) and a protein of unknown function (DRB0099). This operon is strongly induced by ionising radiation [19] and presumably plays a role in DNA strand-break repair (see Table 2). DRB0098 has a very unusual domain structure containing a phosphatase domain of the HD superfamily and a polynucleotide kinase domain [15]. The enzyme possesses 5'-polynucleotide kinase and 3'-phosphatase activities, which are both necessary to seal DNA strand-breaks caused by ionising radiation (Blasius *et al.*, unpublished data).

Some common DNA repair enzymes are even missing in *D. radiodurans*. *D. radiodurans* seems to lack a functional SOS response system as well as genes for UmuC, an error-prone DNA polymerase acting in translesion synthesis, and UmuD that is usually involved in translesion synthesis together with UmuC and RecA [15]. The absence of these enzymes and also of exonuclease I might be important to prevent DNA degradation or the accumulation of mutations [37].

Manganese

D. radiodurans DNA is associated with high levels of Mn(II) [38] and the presence of Mn(II) has been shown to be important for radioresistance [39]. Mn(II) does not directly prevent DNA double-strand breaks [39], but it can mimic the activities of catalase and superoxide dismutase [40]. Recently, Daly *et al*. compared radiosensitive and radioresistant bacteria in respect to their cytosolic ion concentrations. They found that *D. radiodurans* as well as other radioresistant bacteria have high intracellular Mn/Fe concentration ratios and they could show a link between Mn(II) ions and protein protection [41]. Although the idea that superoxide mediated oxidation of proteins is a main cause for radiosensitivity is intriguing, several questions should be addressed in the future to decide which contributions DNA repair and protein protection finally have. The mechanism by which Mn(II) could protect proteins and the reason for cellular ion distribution remain unclear. Mn(II) was found to be globally distributed throughout the cytosol, but X-ray fluorescence micro probe analysis revealed an accumulation of Fe(II) at the septum between dividing cells and high Mn(II) concentrations at

electron-dense granules in the centre of the nucleoids. Furthermore, DNA damage has been displayed only as DNA strand-breaks, whereas DNA cross-links, severe base damage and the resulting toxicity might also be effected by the presence of high Mn(II) concentrations.
Despite the correlation between protein protection and Mn/Fe ratio the role of specialised DNA repair systems in *D. radiodurans* does not need to be marginal.

Nucleoid structure

For many years there has been a lively debate about whether tightly packed and laterally ordered DNA toroids contribute to radioresistance in *D. radiodurans* by facilitating error-free end-joining of double-strand breaks [42]. Donut-like DNA structures were detected by transmission electron microscopy of *in vitro* DNA toroids and restricted diffusion was discussed to be a general strategy for rapid DNA repair upon γ-irradiation [43-45]. Recently, Eltsov and Dubochet performed cryoelectron microscopy using vitreous sections of *D. radiodurans* and were unable to detect any particular order of the DNA. They argue against the presence of DNA toroids and claim that previous results were somehow misinterpreted. Even though the DNA can condense in liquid crystalline phases, DNA ends would remain mobile and compact DNA rings are unlikely to play a key role in radioresistance [46, 47]. Other studies also speak against a direct correlation between radioresistance and ring-like structures: Extremely radioresistant organisms such as *D. radiopugnans* do not display a specific or compact nucleoid shape [48] and radiosensitive bacteria like *E.coli* can display a ring-like nucleoid detectable by transmission electron microscopy and fluorescent microscopy as discussed by Ghosal *et al.* [49]. Nevertheless, restricted diffusion would be a reasonable explanation for efficient repair of double-strand breaks. However, pre-alignment of the chromosome has never been shown and even if a toroidal stucture would exist in *D. radiodurans* it would probably rather be a hindrance for proper DNA repair as DNA repair complexes could have difficulties in reaching the DNA lesions.
To some degree, DNA compaction could be achieved via specialised proteins. *D. radiodurans* encodes various proteins that bind and protect DNA, stabilise repair intermediates or help maintaining the structure of the chromosome. One protein of that kind is Dps-1 (DNA protection during starvation, see Table 2), which as a dimer or dodecamer protects DNA from oxidative damage [50]. The structure of *D. radiodurans* Dps-1 has been solved [51, 52] and the protein possesses a unique N-terminal extension, which is important for DNA-binding and the formation of protein dodecamers. It has been suggested that interaction of two N-termini in successive DNA major grooves would lead to stacked protein-

DNA layers and compaction of the DNA [53]. At least one more Dps protein is encoded by *D. radiodurans* and the gene is induced upon γ-irradiation [8, 19].

The HU-homolog encoded by *D. radiodurans* is probably involved in stabilising DNA repair intermediates and DNA structure in general, but the specific functions of this protein are not yet clear. *D. radiodurans* encodes also an Smc1/Cut3/Cut14 family protein (DR1471), which might be involved in DNA architecture. SMC proteins are conserved in all domains of life [54] and disruption of the SMC gene in *B. subtilis* causes decondensation and missegregation of chromosomes [55], indicating similar or even identical functions of bacterial SMC proteins in comparison to the eukaryotic SMC complexes. Evidence is available that *B. subtilis* SMC has cohesin-like functions and promotes DNA repair [56, 57] and it would be interesting to look closer at the role of *D. radiodurans* SMC in DNA packaging and DNA repair processes.

Concluding remarks and perspectives

An aspect that might play a so far underestimated role is that *D. radiodurans* seems to have a more sophisticated signal transduction system than “normal” bacteria such as *E. coli*. The signal transduction of *D. radiodurans* has features of both prokaryotic and eukaryotic systems and protein-serine/threonine phosphorylation-dependent regulatory pathways are expected to play a major role [15]. *D. radiodurans* also encodes a protein that contains a SARP domain, which in eukaryotes in involved in apoptosis-related signalling [58]. Irradiated *D. radiodurans* cultures show a dose dependent growth lag. This inhibition of replication is similar to eukaryotic DNA damage checkpoints that allow time for DNA repair and “cell cleaning”. Some indications for bacterial checkpoints have been observed, like cell-division inhibitors in *E. coli* [59] or a checkpoint for sporulation in *B. subtilis* [60], and are frequently discussed. Nevertheless a bacterial checkpoint has not yet been formally established. There are also hints that *D. radiodurans* tightly regulates transcription, which might help to respond efficiently to stress conditions. A family of predicted HTH regulators is expanded and suggests a role in the regulation of a distinct set of genes. Several members of this family are upregulated upon ionising radiation and one of them appears to be associated with γ-resistance [61, 62]. Probably strict regulation occurs additionally via small non-coding (sn) RNA. A role for these short RNA molecules in bacterial gene expression has been proposed recently [63]. Interestingly, three extremely radioresistant strains of *E.coli* K12 could be evolved through several selection rounds with high doses of ionising radiation (Battista *et al.*, submitted). None of those strains showed a change in Mn(II) metabolism or DNA structure and complete genome re-sequencing displayed no overlap in genetic alterations.

Taken together, a single key to radioresistance, be it a special DNA repair system, a high Mn/Fe ratio or a condensed nucleoid, does not seem to exist and further work has to be done to understand the full intricacy of radioresistance. A combination of many factors collectively contributes to a radioresistant phenotype and allows for radioresistance of *D. radiodurans* by various means and mechanisms.

Table 1 **Deinococcaceae**

Species	**Identified in**	**Reference**
D. apachensis	Sonoran desert	[64]
D. deserti	Sahara desert	[65]
D. ficus	Ficus rhizosphere	[66]
D. frigens	Antarctica	[67]
D. geothermalis	Hot springs, Italy and Portugal	[68]
D. grandis	Intestine of Cyprinus carpio	[69]
D. hohokamensis	Sonoran desert	[64]
D. hopiensis	Sonoran desert	[64]
D. indicus	Aquifer, West Bengal, India	[70]
D. maricopensis	Sonoran desert	[64]
D. marmoris	Antarctica	[67]
D. murrayi	Hot springs, Portugal	[68]
D. navajonensis	Sonoran desert	[64]
D. papagonensis	Sonoran desert	[64]
D. peraridilitoris	coastal desert, Chile	[71]
D. pimensis	Sonoran desert	[64]
D. proteolyticus	Faeces of Lama glama	[72]
D. radiodurans	Irradiated meat cans	[1]
D. radiophilus	Irradiated Bombay duck	[72]
D. radiopugnans	Irradiated Haddock	[72]
D. saxicola	Antarctica	[67]
D. sonorensis	Sonoran desert	[64]
D. yavapaiensis	Sonoran desert	[64]
D. yunnanensis	Agar plate, China	[73]
T. radiovictrix	Hot springs, Azores	[74]

Table 2 ***D. radiodurans*** **genes involved in DNA protection and DNA repair**

Gene	Protein	Function	Special characteristics	Reference
DR0099	DraSSB	protection of single-stranded DNA	functions as dimer, each monomer has two OB-folds	[34, 36, 75, 76]
DR0198	RecR	daughter-strand gap repair, repair of interstrand crosslinks	Topprim domain, forms tetramer that builds a DNA clamp	[30, 31]
DR0423	DdrA	protection of 3' ends of single-stranded DNA	strongly induced upon IR, homolog of Rad52	[8, 16]
DR0467	$PolX_{Dr}$	DNA polymerase/ 3'-5' exonuclease	nuclease is structure-modulated, uses Mn(II)	[25, 26]
DR0690	DraTopIB	DNA topoisomerase	related to poxvirus and eukaryotic-type DNA topoisomerases	[19, 77-79]
DR0819	RecO	DNA annealing and RecA-mediated recombination	similarities to eukaryotic proteins RP-A and BRCA2	[32, 80]
DR1289	RecQ	DNA helicase	three tandem HRDC domains regulate enzymatic activity	[81-83]
DR1707	Pol I	DNA repair, TLS?	modulated by Mn(II)	[10, 84]
DR1751	UNG	Uracil-DNA glycosylase	preference for single-stranded DNA, member of class 4 family	[85, 86]
DR1902	RecD	DNA helicase superfamily I	involved in antioxidant pathway and resistance, not part of RecBCD	[28, 29, 87]
DR2263	Dps-1	DNA protection during starvation, ferroxidase	N-terminal extension that is essential for DNA binding and dodecamer assembly	[50-53]
DR2340	$RecA_{Dr}$	homologous recombination	inverse DNA strand exchange pathway	[11, 12, 88-90]
DRA0065	HU	stabilisation of DNA junctions?	different DNA binding specificity than *E.coli* HU, no architectural role	[91, 92]
DRA0346	PprA	DNA binding and tethering of termini, stimulation of DNA ligases and catalase	unique protein, under control of a radiation responsive promoter	[8, 17, 18, 93, 94]
DRB0098	PNKP	polynucleotide kinase/ 3' phosphatase	unique domain architecture, Mn(II) as preferred cofactor	[15, 19]

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5.5. DNA repair enzymes characterised in this work

Deoxyribonucleases, reviewed in [31, 32], catalyse the hydrolysis of phosphodiester bonds between deoxyribonucleotides. Exonucleases remove one or several nucleotides from the end of a DNA chain, moving either in 3'-5' or in 5'-3' direction. Few exonucleases can act both from the 3' as well as from the 5' end of a DNA strand. Deoxyribonucleases exist either alone or in association with a Pol (so called proofreading nucleases, e.g.Pol I) or a DNA helicase (e.g. Dna2) [33]. They are involved in many pathways such as DNA replication, DNA recombination, DNA repair and degradation of defective DNA. Deoxyribonucleases can degrade ss DNA, ds DNA or both of them. Both prokaryotes and eukaryotes contain many different exonucleases that are active in different processes, and often one nuclease can perform in diverse repair systems so that redundancy is ensured in each cell. Several nucleases are modulated by specific DNA structures [34]. Examples are the bacterial SbcCD nuclease active on hairpin DNA [35] or the eukaryotic flap endonuclease 1 involved in replication and base excision repair [36].

Pols (reviewed in [37]) are important for replication, recombination and repair of DNA. They catalyse the polymerisation of deoxyribonucleotides. Most Pols need a template and an RNA- or DNA primer that can be elongated in 5'-3' direction. Some Pols are associated with a 5'-3' proofreading exonuclease for increased fidelity. All Pols can be grouped into seven different families based on their sequence homologies: Pols from the A, B, C and D families are mainly replicative, whereas those from the families X and Y are involved in DNA repair and translesion synthesis, respectively. Reverse transcriptase represents a separate class of Pols, which is present in retroviruses and uses viral RNA as a template [37].

E. coli has 5 Pols named Pol I, Pol II, Pol III, Pol IV and Pol V. Pol III is the replicative polymerase, whereas Pol I and Pol II are implicated in DNA repair and replication of damaged DNA. Pol I, Pol II and Pol III are all associated with 3'-5' exonuclease activities. *E. coli* encodes 2 additional Pols belonging to the Y-family [38]. Pol IV (DinB) and Pol V (UmuC/UmuD), are required for error-prone translesion synthesis. Interestingly, *D. radiodurans* does not encode genes for Pol IV or PolV [1].

The Pol family X contains several well-known eukaryotic DNA polymerases such as DNA Pol β, Pol λ, Pol μ, and terminal deoxyribonucleotidyltransferase. Pol β is implicated in base-excision repair and Pol λ in non-homologous end-joining, translesion synthesis and base-excision repair [39]. Recently, several bacterial and archaeal family X members have been identified, often in combination with a polymerase- and histidinol-phosphatase domain and

the proteins have been predicted to be involved in DNA repair. However, so far none of these prokaryotic proteins has been characterised biochemically.

DNA ligases (reviewed in [40]) are present in all cellular organisms. They also take part in essential processes, such as DNA replication, recombination and DNA repair. DNA ligases catalyse the formation of a phosphodiester bonds between 3'-OH and 5'-phosphate termini. NAD^+-dependent DNA ligases are exclusively found in prokaryotes, whereas ATP-dependent DNA ligases are present in eukaryotes, viruses, archaea and several bacteria. All DNA ligases contain several conserved motifs, especially a highly conserved KxDG sequence. In a first step, the conserved lysine residue of this motif is adenylated by the AMP group of either NAD^+ or ATP. The AMP group is then transferred to the 5'-phosphate end of the DNA. Finally, a nucleophilic attack by the 3'-OH on the activated phosphorus occurs and the leaving group AMP is released. The result is a new 3'-5'-phosphodiester bond.

Polynucleotide kinases [41, 42] transfer the γ-phosphate group of ATP to the 5' end of a polynucleotide that lacks a 5'-phosphate. Polynucleotide kinases are usually also 3'-phosphatases and both enzymatic activities are harboured in separate domains of the same polypeptide. 3'-phosphatases catalyse the hydrolysis of 3'-phosphoryl groups of DNA termini 3'-phosphoryl nucleotides. Both of these activities are required to prepare DNA strand-breaks for subsequent ligation. Ionising radiation is known to cause DNA strand-breaks that often lack a 5'-phosphate or that are phosphorylated at the 3'-terminus.

Table 1l **Selected genes from *D. radiodurans***

Protein name	Gene	Function	Fold induced upon IR[1]	Mutant Phenotype	Reference
DdrC	DR0003	unknown function	14/12	radiosensitive in ΔRecA	[15, 26, 43]
-	DR0022	Uracil-DNA glycosylase	3.33	n.d.	[26, 44]
-	DR0052	unknown function	6.5/5	n.d.	[15, 26]
-	DR0053	DinB/YfiT family	10/5	n.d.	[15, 26]
DdrB	DR0070	alkaline phosphatase?	4/42	radiosensitive	[15, 26, 43]
-	DR0140	unknown function	6	radioresistant	[26]
DdrD	DR0326	unknown function	11	radiosensitive in ΔRecA	[15]
GyrB	DR0906	DNA gyrase, subunit B	4.41/8	n.d.	[15, 26]
-	DR1142	unknown function	10/5	n.d.	[15, 26]
Rsr	DR1262	Ro ortholog, RNA quality control?	1.04/4	UV sensitive	[26, 29, 30]
DdrM	DR1440	unknown function	10	n.d.	[15]
RecN	DR1477	DNA repair protein, ATP-binding	0.93	general repair deficiency	[26, 45]
-	DR1721	3'-5' exonuclease	1.36	n.d.	[6, 26]
-	DR1757	predicted nuclease with zinc finger domain	1	n.d.	[6, 26]
UvrD	DR1775	superfamily II helicase	3	mutator phenotype, less competent	[26, 46, 47]
-	DR1901	unknown function	11	n.d.	[26, 43]
GyrA	DR1913	DNA gyrase, subunit A	3.29/13	reduced viability, aberrant morphology	[15, 26, 48]
RecG	DR1916	superfamily II helicase	3	radioresistant	[26, 43]
LigA	DR2069	NAD^+-dependent DNA ligase	0.2	likely to be essential	[26], this work
-	DR2097	conserved protein of unknown function	7	n.d.	[26]
LigT	DR2339	2'-5' RNA ligase	14/6	radiosensitive	[15, 26]
-	DR2441	acetyltransferase?	2.6/9	n.d.	[2, 15, 26, 43]
-	DR2444	RecQ helicase?	2.43	n.d.	[6, 26]
DdrO	DR2574	HTH transcription factor, phage type	5/8	n.d.	[15, 26, 43]
-	DRB0092	Dps/ferritin family	3/8	n.d.	[15, 26]
DraRnl	DRB0094	RNA ligase	3	n.d.	[26, 28, 49]
-	DRB0099	putative Macro domain family member	9	n.d.	[9, 26, 43] this work
-	DRB0100	putative ATP-dependent DNA ligase	14	radioresistant	[15, 26, 43] this work

[1] Two different values indicate the result from two different studies. The first number refers to the work of Liu *et al*. (2003), whereas the second refers to Tanaka *et al*. (2004).

5.6. Aim of this thesis

The aim of this thesis was to identify and characterise some novel DNA repair enzymes from *D. radiodurans* in order to better understand the basic requirements for radioresistance. Factors that seem to be important for radioresistance are further discussed in a review in 5.4., and DNA repair enzymes are clearly contributing to this phenotype. However, many of the basic DNA repair enzymes from *D. radiodurans* have not been characterised so far.

In this thesis work, a prediction-driven approach has been applied: Gene functions can be predicted based on sequence alignments with homologs found in other organisms. The proteins are then expressed in *E.coli*, purified and enzymatically characterised *in vitro*. Deletion of the gene in *D. radiodurans* and analysis of the resulting phenotype can provide additional information on the relevance of the corresponding DNA repair pathway. The first protein identified and characterised was a family X Pol. The gene has been identified based on sequence homology to human Pols β and λ and surprisingly turned out to be an efficient structure-modulated nuclease. Other proteins characterised in this work are a DNA ligase and a polynucleotide kinase, both of which are likely to play major roles in the repair of DNA strand breaks.

6 ORIGINAL RESEARCH ARTICLES

6.1 DNA polymerase X from *Deinococcus radiodurans* possesses a structure-modulated 3'-5' exonuclease activity involved in radioresistance

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DNA polymerase X from *Deinococcus radiodurans* possesses a structure-modulated 3′→5′ exonuclease activity involved in radioresistance

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Summary

Recently a family X DNA polymerase ($PolX_{Dr}$) was identified in the radioresistant bacterium *Deinococcus radiodurans*. Knockout cells show a delay in double-strand break repair (DSBR) and an increased sensitivity to γ-irradiation. Here we show that $PolX_{Dr}$ possesses 3′→5′ exonuclease activity that stops cutting close to a loop. $PolX_{Dr}$ consists of a DNA polymerase X domain (PolXc) and a Polymerase and Histidinol Phosphatase (PHP) domain. Deletion of the PHP domain abolishes only the structure-modulated but not the canonical 3′→5′ exonuclease activity. Thus, the exonuclease resides in the PolXc domain, but the structure-specificity requires additionally the PHP domain. Mutation of two conserved glycines in the PolXc domain leads to a specific loss of the structure-modulated exonuclease activity but not the exonuclease activity in general. The PHP domain itself does not show any activity. $PolX_{Dr}$ is the first family X DNA polymerase that harbours an exonuclease activity. The wild-type protein, the glycine mutant and the two domains were expressed separately in Δ$polX_{Dr}$ cells. The wild-type protein could restore the radiation resistance, whereas intriguingly the mutant proteins showed a significant negative effect on survival of γ-irradiated cells. Taken together our *in vivo* results suggest that both $PolX_{Dr}$ domains play important roles in DSBR in *D. radiodurans*.

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Introduction

Deinococcus radiodurans is a highly radioresistant bacterium that can survive hundreds of double-strand breaks (DSB) (Battista *et al*., 1999; Cox and Battista, 2005). The *D. radiodurans* genome encodes the majority of prokaryotic repair genes, but the molecular mechanisms for its extraordinary radioresistance are not yet fully understood. It is believed that genome restitution in *D. radiodurans* γ-irradiated cells is mediated by RecA-dependent homologous recombination, although DNA repair via RecA-independent pathways may also take place (Cox and Battista, 2005).

Family X DNA polymerases play important roles in different DNA repair processes (Ramadan *et al*., 2004). Recently, we showed that the *D. radiodurans* gene DR0467 encodes a family X polymerase ($PolX_{Dr}$) which possesses Mn^{2+}-dependent polymerase activity (Lecointe *et al*., 2004). The preference for Mn^{2+} as a cofactor matches the finding that *Deinococcus* has very high intracellular Mn^{2+} levels (Daly *et al*., 2004). Knocking out $PolX_{Dr}$ leads to a significant delay in double-strand break repair (DSBR) as well as to an increased sensitivity to γ-irradiation (Lecointe *et al*., 2004).

In this work we show that $PolX_{Dr}$, in addition to its polymerase activity, has a strong Mn^{2+}-dependent 3′→5′ exonuclease activity that is located in the polymerase domain and that it specifically recognizes and pauses at stem loops. Exonucleases may play important roles in DNA repair by processing damaged DNA or repair intermediates thus generating substrates for DNA polymerases and DNA ligases. Neither a standard nor a structure-modulated 3′→5′ exonuclease activity has been observed so far for a family X DNA polymerase, even though other polymerases are often associated with 3′→5′ exonucleases that act as proof-readers during DNA replication, repair and recombination (Shevelev and Hubscher, 2002).

By expressing mutant $PolX_{Dr}$ proteins in Δ$polX_{Dr}$ cells, we also show that the $PolX_{Dr}$ stem-loop 3′→5′ exonuclease activity is required for efficient *in vivo* repair of DSB. The exonuclease activity of the Deinococcal DNA polymerase may play important roles in DNA repair by processing damaged DNA or repair intermediates thus generating substrates for other repair proteins.

Results

The PolX$_{Dr}$ protein harbours a nuclease activity

The product of the *PolX$_{Dr}$* gene was expressed with a N-terminal His-tag as described before (Lecointe *et al.*, 2004). PolX$_{Dr}$ showed a measurable polymerase activity. In addition, a strong Mn^{2+}-dependent nuclease activity co-purified with PolX$_{Dr}$ through all steps of purification. To show that this activity was intrinsic to PolX$_{Dr}$, two approaches were used. First, a gel filtration on a Superose™ 12 column (Fig. 1A) was performed, where PolX$_{Dr}$ exactly co-eluted with a strong nuclease activity. The presence of PolX$_{Dr}$ was confirmed by Western blot using affinity-purified rabbit IgG against recombinant PolX$_{Dr}$. Second, we performed *in situ* nuclease activity gels using [^{32}P]-labelled ϕX174 DNA (Fig. 1B) with either His-tagged or MBP-tagged PolX$_{Dr}$. For this experiment, proteins are loaded on a SDS gel that contains ^{32}P-labelled DNA. The proteins are then renatured *in situ* and incubated under the conditions used for a nuclease assay in which the nuclease is detected as a light band on an autoradiograph. As a positive control DNase I was used (Fig. 1B, lane 1). All proteins gave signals at their expected molecular weight positions. No other nuclease bands could be detected for both PolX$_{Dr}$ protein preparations ultimately excluding the possibility of contaminants from *Escherichia coli*. So far, no known *E. coli* nuclease shows similar strong Mn^{2+}-dependence like PolX$_{Dr}$. The biochemical properties of the PolX$_{Dr}$ nuclease are summarized in Table 1.

PolX$_{Dr}$ is a 3′→5′ exonuclease

To further characterize the PolX$_{Dr}$ nuclease, we labelled a 25mer single-strand oligonucleotide on the 3′ end. As the 3′ labelling was done by terminal transferase, the 3′-labelled DNA substrate had a size of 26–27 nucleotides (nt). Time-course experiments clearly indicated that PolX$_{Dr}$ possesses a 3′→5′ exonuclease activity because an immediate 1-nt product appeared (Fig. 2A). PolX$_{Dr}$ was active either on linear single-strand or double-strand DNA (Fig. 2B). The digestion pattern when the DNA substrate was labelled at 5′ end (Fig. 2B) was similar to Trex1, a well-characterized 3′→5′ exonuclease (Mazur and Perrino, 2001).

The PolX$_{Dr}$ 3′→5′ exonuclease is modulated by the DNA structure

We noticed that PolX$_{Dr}$ exhibits an exonuclease activity on oligonucleotides with a potential to form hairpins or loops but strongly pauses as soon as the loop is at a close distance (Fig. 3B). In fact, we first tested an oligonucleotide with a stable stem of 20 nt and a loop of 5 nt. The main digestion product showed a size of ≈39 or 40 nt, suggesting a pausing site 5 to 6 nt from the 3′ end of the 45mer oligonucleotide ('loop DNA-3' in Fig. 3A). The pattern did not change when the loop was hybridized to either a linear oligonucleotide, thus forming double-strand DNA on each side of the stem ('loop DNA 2' in

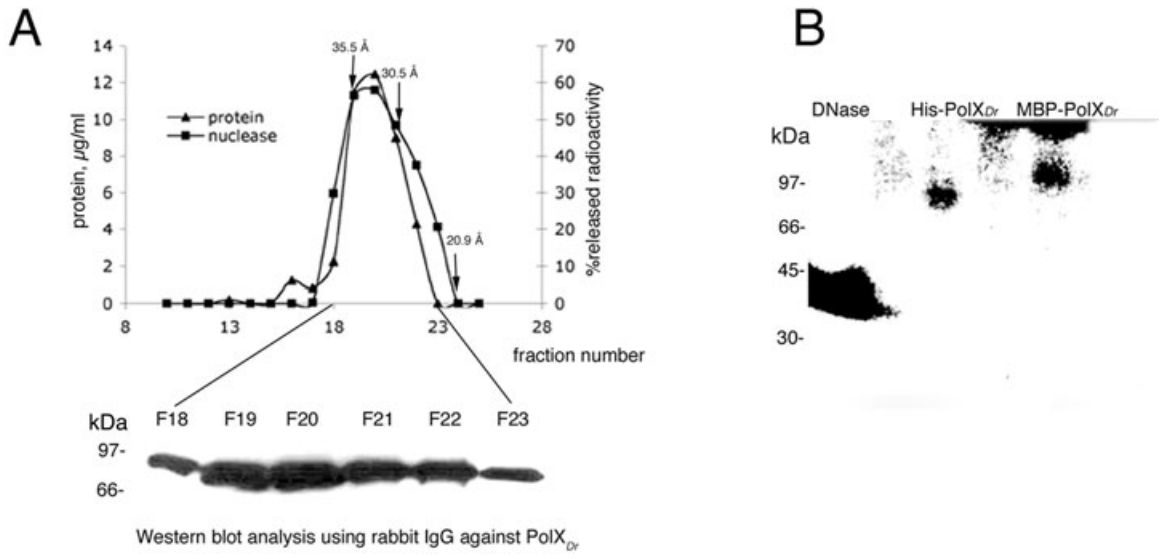

Fig. 1. The nuclease activity is an intrinsic property of the PolX$_{Dr}$ polypeptide.
A. Purified His-tagged PolX$_{Dr}$ was loaded on a Superose™ 12 gel filtration column and the nuclease activity of the eluted fractions was determined as described in *Experimental procedures* using [^{3}H]-labelled DNA. The stokes radius for protein markers is indicated at the corresponding positions. The eluted protein peak was additionally confirmed by Western blot using IgG against His-tagged PolX$_{Dr}$.
B. Nuclease *in situ* activity gel using [^{32}P]-labelled DNA. The DNA was included in the gel mix and nucleases can be identified as light bands after autoradiography. The picture was 'inverted' in Photoshop for better illustration. As a positive control 50 ng of DNase I was loaded (lane 1). Two hundred and fifty nanograms of His-tagged PolX$_{Dr}$ (lane 3) or 500 ng of MBP-tagged PolX$_{Dr}$ (lane 5) were tested.

Table 1. Optimal reaction conditions for the $PolX_{Dr}$ nuclease[a].

Reaction conditions	Relative nuclease activity
pH 8.0	1
pH 6.8	0.94
pH 9.0	0.6
EDTA, 10 mM	0.06
$MgCl_2$, 5 mM	0.08
NaCl, 100 mM	0.43
ATP, 1 mM	0.42
dATP, 1 mM	0.55

a. Reactions were carried out in a final volume of 10 µl containing: 10 ng Pol X_{Dr}, 160 fmol [^{3}H]-labelled activated DNA (≈ 2000 cpm), 1 mM $MnCl_2$ (with the exception when EDTA and MgCl2 were tested), 40 mM Tris/HCl pH 8 (unless otherwise mentioned). The reactions were incubated for 15 min at 37°C and the products were analysed as outlined in *Experimental procedures*. Maximal activity represents 55% released radioactivity.

Fig. 3A) or to a circular M13 DNA ('loop DNA 1' in Fig. 3A). Next we tested whether the loop size had an influence on the pausing site. For this, two additional DNA substrates (Fig. 4A) with a bigger loop of 10 or 15 nt, respectively, were prepared. Time-course experiments with $PolX_{Dr}$ showed no substantial difference for the three substrates tested, suggesting that the loop size itself has no influence on the pausing site located in the stem (Fig. 4B).

We next checked whether the specific pausing site depends on the polarity of the DNA strand or on the DNA sequence. For this, we compared the 'loop DNA-3' (Fig. 3A) with the 'loop DNA 3-Anti', which has the same structure but a complementary stem sequence (see also Table 2). Both DNA substrates were cut in the stem 3′ to the loop (Fig. 5A), as confirmed by digesting the same oligonucleotides with S1 nuclease which can only cut in the loop, thus producing smaller products (data not shown). In the case of $PolX_{Dr}$, the main product with 'loop DNA 3-Anti' was migrating slower than the product of the 'loop DNA-3'. This might be due to the different mass of the products or due to an influence of the sequence on the exact cutting position. As in both cases the cutting was occurring 3′ of the loop, we suggest that the polarity of the DNA strand determines the cutting. To exclude an additional endonuclease activity, we used the oligonucleotide 'loop DNA 3-Anti' with a ^{32}P-label located on the 3′ end. In this case, a single nucleotide product appeared right away (Fig. 5B).

To further analyse the substrate specificity of $PolX_{Dr}$, we also incubated $PolX_{Dr}$ with double-strand DNA oligonucleotide of 25 nt length containing two unpaired nucleotides at positions 12 and 13 (see Table 2). $PolX_{Dr}$ cuts the oligonucleotide as a 3′→5′ exonuclease without a significant pausing site (Fig. 5C). Finally we also tested $PolX_{Dr}$ on an oligonucleotide of 39 nt with a 3′-flap of 20 nt length, a standard substrate for the flap-endonuclease Fen1 (Friedrich-Heineken *et al*., 2003). Again a digestion pattern characteristic for a 3′→5′ exonuclease was evident (Fig. 5D, lanes 1–4). To confirm the correct structure of the DNA, we performed a control reaction using 5 ng His-Fen1, which was prepared as published before (Friedrich-Heineken *et al*., 2003). Fen1 cuts the oligonucleotide as expected giving products of 20 and 21 nt length (Fig. 5D, lane 6). In summary, our results clearly suggest that the $PolX_{Dr}$ exonuclease is a 3′→5′ exonuclease that is modulated by stem loops.

The $PolX_{Dr}$ polymerase domain alone is a canonical 3′→5′ exonuclease only

$PolX_{Dr}$ contains two domains, an N-terminal catalytic domain, PolXc, homologous to eukaryotic pols from the X family and a C-terminal histidinol phosphatase domain (PHP) that may be involved in amino acid transport and metabolism. An identical architecture is observed in homologues from *Bacillus subtilis, Methanothermobacter thermoautotrophicum* and from most of bacterial species containing a putative $PolX_{Dr}$ homologue (Lecointe *et al*.,

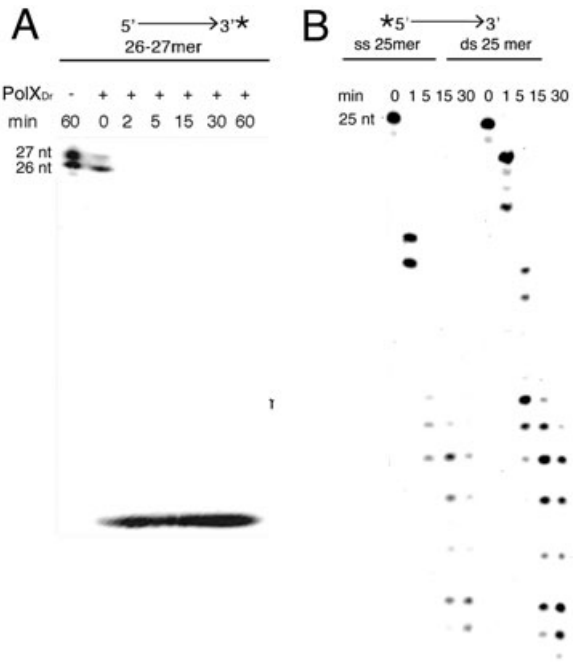

Fig. 2. $PolX_{Dr}$ is a 3′→5′ exonuclease acting on single-strand (ss) and double-strand (ds) DNA.
A. Reactions were carried out as described in *Experimental procedures* with 50 ng of $PolX_{Dr}$ and 50 fmol 3′-labelled oligonucleotide. Samples were taken at different time points and products were analysed on a 15% denaturing polyacrylamide gel and visualized by autoradiography. Oligonucleotides are schematically presented on top of the panel and their sequences are shown in Table 2.
B. Fifty nanograms of $PolX_{Dr}$ were incubated with 50 fmol either single-strand or double-strand 5′-labelled oligonucleotides of 25 nt length for the indicated time points. Products were analysed on a denaturing 18% polyacrylamide gel.

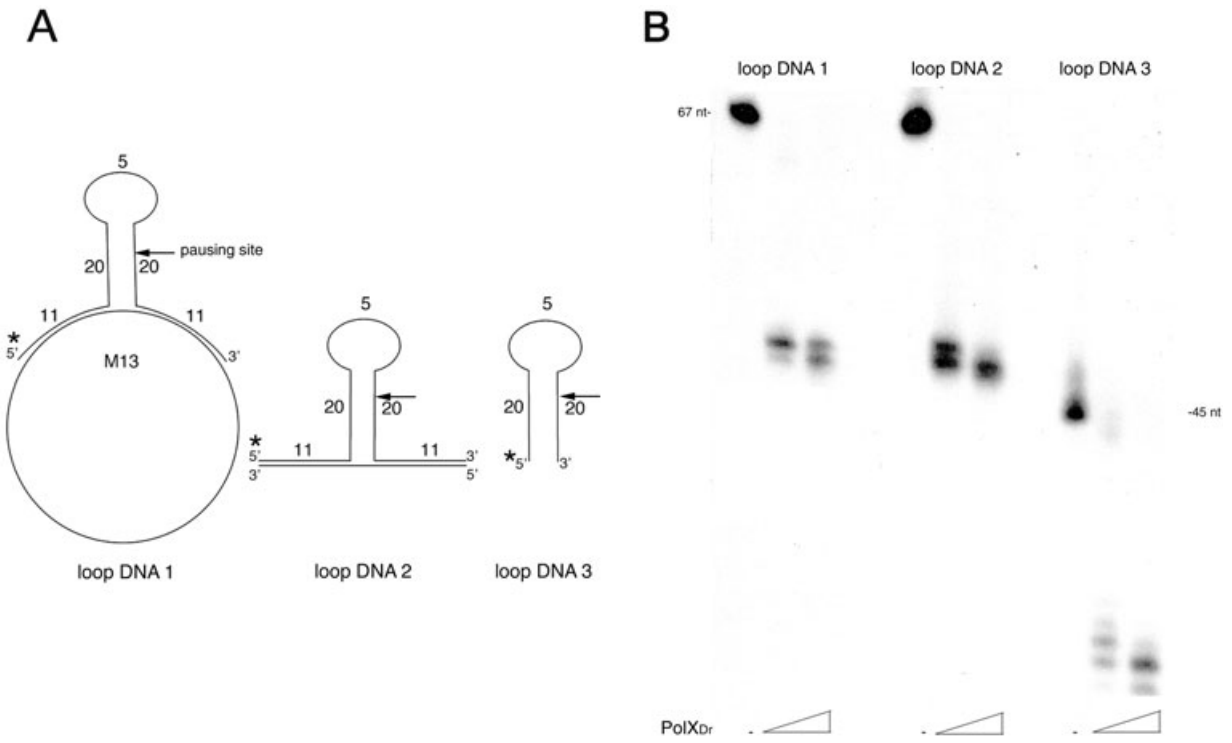

Fig. 3. PolX$_{Dr}$ stops cutting 5′-labelled stem-loop substrates 3′ of the loop in the stem.
A. Schematic representation of the stem-loop oligonucleotides used in (B). *, position of the [^{32}P]-label. For oligonucleotide sequences see Table 2. Arrows indicate the approximate pausing sites of the PolX$_{Dr}$ exonuclease.
B. 5′-labelled oligonucleotides were incubated for 30 min at 37°C with either 25 or 50 ng of PolX$_{Dr}$. Reaction products were analysed by electrophoresis on a denaturing 15% polyacrylamide gel and subsequent autoradiography.

Table 2. Oligonucleotides used in this study to characterize the nuclease activity[a].

DNA substrate[b]	DNA used	Sequence in 5′→3′ direction	Length (nt)
Activity gel DNA	ϕX174 DNA	See gi216019 in the NBCI DNA database	5386
	Primer 1	GGAAAGCGAGGGTAT	15
Single-strand 25mer (Fig. 2)	25mer	GGTGAAGAAGGACGAGGAGCTGAGC and complementary oligonucleotide for double-strand 25mer	25
Loop DNA 1	M13mp2 DNA	See gi310751 in the NBCI DNA database	7243
	LoopM13	CGATCGGTGCGGGGGGGGTTGAAGGGGGGGGAAAAACCCCCCCCTT CAACCCCCCCGGGCTCTTCGC	67
Loop DNA 2	Loop for M13 DNA	CGATCGGTGCGGGGGGGGTTGAAGGGGGGGGAAAAACCCCCCCCTT CAACCCCCCCGGGCTCTTCGC	67
	Linear M13 fragment	GCGAAGAGGCCCGCACCGATCG	22
Loop DNA 3	Loop DNA 3	CCCCCCCAACTTCCCCCCCCAAAAAGGGGGGGGAAGTTGGGGGGG	45
Loop DNA 3-Anti	Loop DNA 3-Anti	GGGGGGGTTGAAGGGGGGGGAAAAACCCCCCCCTTCAACCCCCCC	45
Loop DNA 3–10 nt loop	Loop DNA 3–10 nt loop	CCCCCCCAACTTCCCCCCCCAAAAAAAAAAGGGGGGGGAAGT TGGGGGGG	50
Loop DNA 3–15 nt loop	Loop DNA 3–15 nt loop	CCCCCCCAACTTCCCCCCCCAAAAAAAAAAAAAAAGGGGGGGGAAGTT GGGGGGG	55
2-Mismatch DNA	494F	GCGGTGCTCTTGGTGGCGCGAAACC	25
	494R	GGTTTCGCGCCAGGAAGAGCACCGC	25
3′-Flap-DNA	UP1	TCGAGGTCGACGGTATCGATAAGCTTGATA	30
	Dn5	GTCATGATAGATCTGATCGCTCGAATTCCTGCAGCCTGCAGCCCGGCC	39
	T	GGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGA	49

a. DNA substrates that should have defined secondary structures were checked on a 10% native polyacrylamide gel as well as by the structure prediction program MFOLD (Zuker, 2003).
b. Name as used in the text.

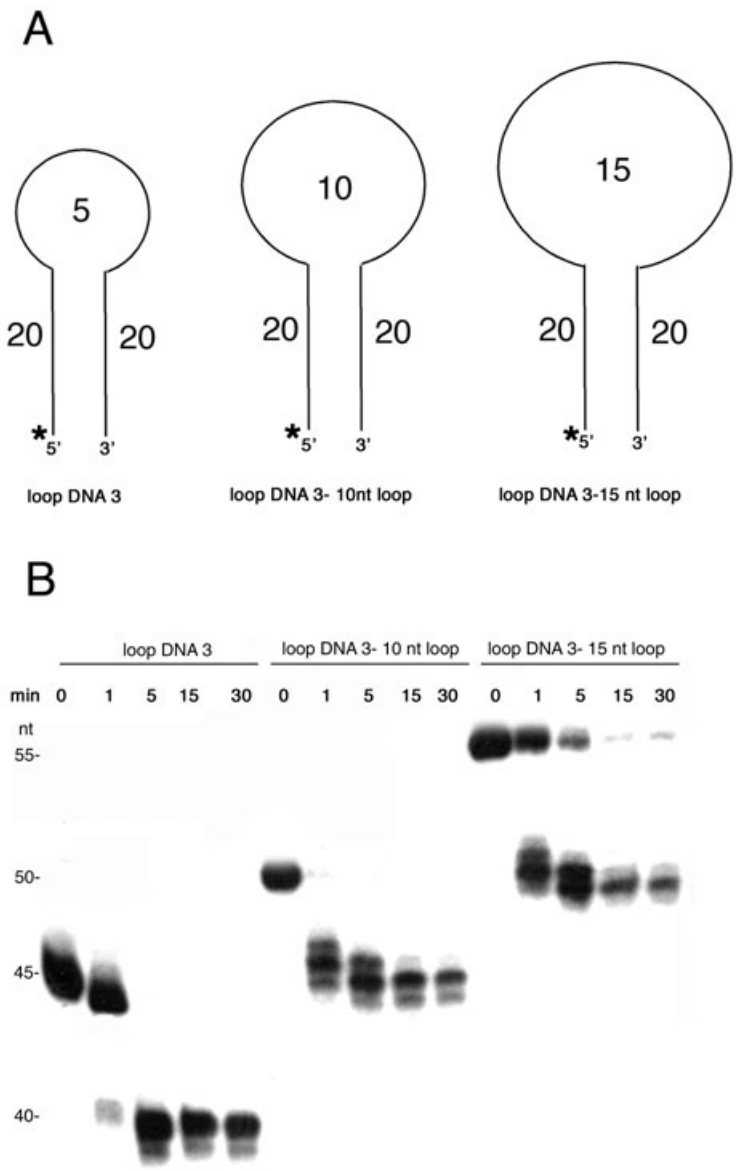

Fig. 4. The loop size has no influence on the pausing position.
A. Schematic representation of the stem-loop oligonucleotides used in (B). *, position of the [^{32}P]-label. The stem part of the oligonucleotides is the same for all three oligonucleotides and the loops contain 5, 10 or 15 adenosyl residues.
B. Three DNA substrates were incubated with 50 ng of PolX$_{Dr}$ and reactions were stopped at different time points. Products were analysed by electrophoresis on a denaturing 15% polyacrylamide gel and visualized by autoradiography.

2004). These domains do not contain a canonical nuclease motif. In order to map the nuclease activity to one of the two domains, we expressed the two domains separately and tested them for nuclease activity. The PolXc domain alone was poorly expressed in *E. coli* (maximum yield was 20 μg from 1 l bacterial culture). In contrast, the PHP domain was well expressed in *E. coli,* but did not show any nuclease activity. When we analysed the PolXc domain, a band at the expected position of 34 kDa was detected by an *in situ* nuclease activity gel (Fig. 6), suggesting that the nuclease activity is located in the PolXc domain. Neither the Pol Xc nor the PHP domain showed structure-dependent exonuclease activity (data not shown), suggesting that the entire PolX$_{Dr}$ is required for structure recognition.

Mutations of two conserved glycines 104 and 106 leads to loss of the loop specificity but not of the 3′→5′ exonuclease activity itself

To further analyse the structure-modulated nuclease activity, we next created a nuclease mutant PolX$_{Dr}$. For lack of a conserved nuclease motif, we generated a double mutant exchanging the conserved glycines 104 and 106 in the PolXc domain for valines. These two glycines are part of a GXG motif present in all family X DNA polymerases and they have been shown to act as a DNA ligand (Oliveros *et al*., 1997). Both wild type and mutant PolX$_{Dr}$ were purified the same way to homogeneity and the proteins were tested for 3′→5′ exonuclease activity. The mutant protein showed first, a reduced 3′→5′ exonu-

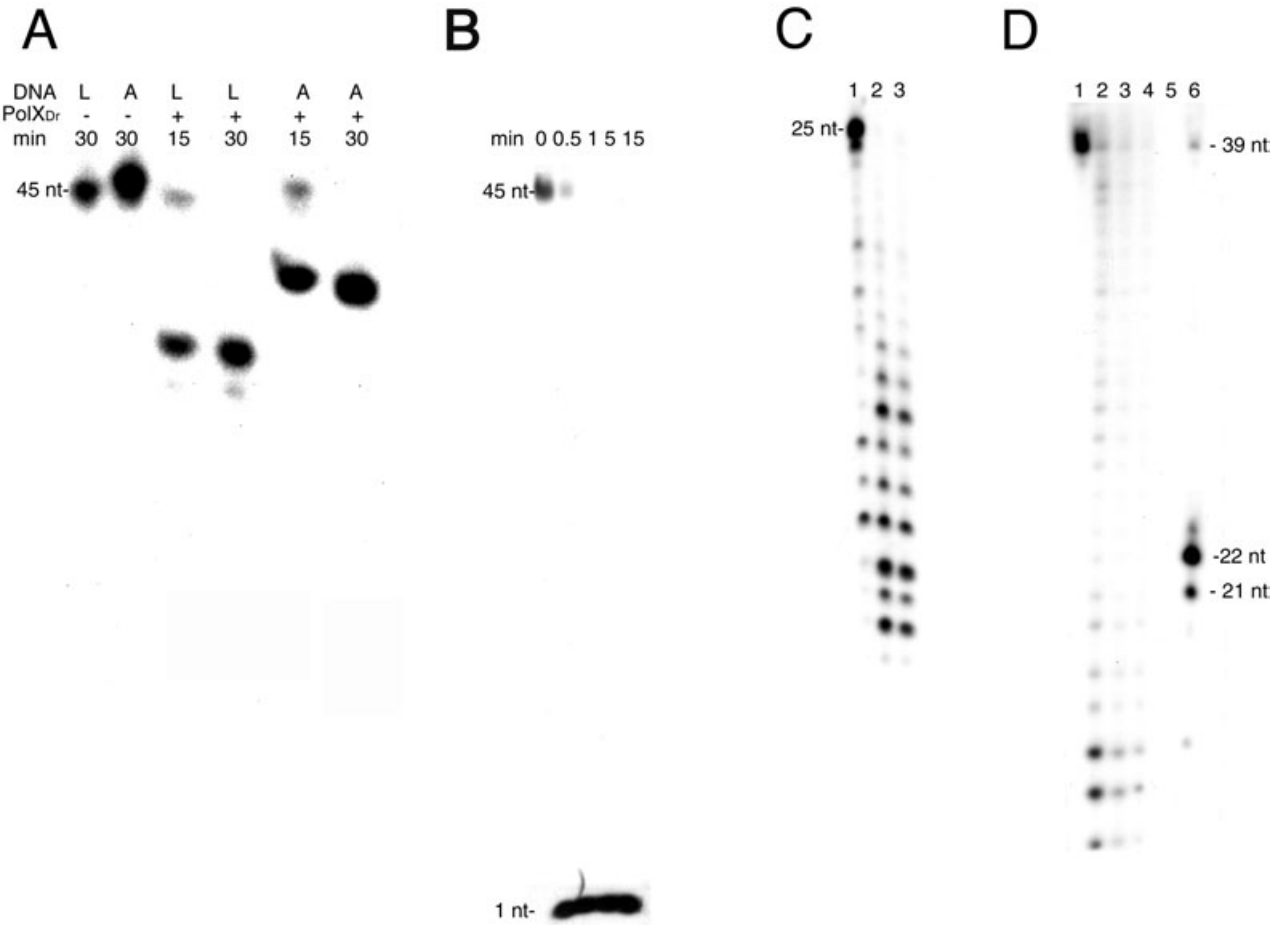

Fig. 5. The pausing site 3′ of the loop is sequence-independent.
A. 25 fmol of the two different DNA substrates loop DNA 3 (L) and loop DNA 3-Anti (A) (see Table 2) were incubated with 50 ng His-PolX$_{Dr}$ for 15 or 30 min. Products were separated on a denaturing 15% polyacrylamide gel and visualized by autoradiography.
B. 100 fmol of loop DNA 3 labelled at the 3′ end was incubated with 25 ng of His-PolX$_{Dr}$ for 0, 0.5, 1, 5 and 15 min and the products were separated on an 18% polyacrylamide gel. Note the immediate appearance of a 1 nt band.
C. 50 fmol of 2-mismatch DNA (see Table 2) were incubated with 0, 25 and 50 ng His-PolX$_{Dr}$ (lanes 1, 2 and 3 respectively) for 30 min. Products were separated on an 18% polyacrylamide gel.
D. 50 fmol of Flap-DNA (see Table 2) were incubated with 0, 25, 50 or 75 ng of His-PolX$_{Dr}$ (lanes 1–4) or 5 ng of His-Fen1 (lane 6). Products were separated on an 18% polyacrylamide gel.

clease activity on homopolymeric DNA oligonucleotide and second, a complete loss of the pausing site on the stem-loop oligonucleotide (Fig. 7). At a protein amount where the wild type and the mutant enzyme show a similar exonuclease activity on the homopolymer (100 ng of wild type and 150 ng of mutant protein), no product could be seen when the mutant enzyme was tested with the stem-loop oligonucleotide (Fig. 7), suggesting that the stem-loop structure could not be cut at all by the double mutant protein. Up to 500 ng of the mutant protein could not cut the stem-loop structure (data not shown).

The PolXc and the PHP domains are required for radioresistance

We previously showed that the expression of the *polX*$_{Dr}$ gene under the control of an inducible promoter restored the γ-ray resistance of a Δ*polX* host to the wild-type level (Lecointe *et al.*, 2004). We used this complementation assay to determine whether the nuclease activity associated with PolX$_{Dr}$ is required for radioresistance and DSBR. We tested three mutant proteins: PolXc and PolX$_{G104VG106V}$ both retaining 3′→5′ exonuclease activity but not being modulated by a stem-loop structure anymore, and PolX$_{PHP}$ devoid of nuclease and polymerase activities. We verified by immunoblotting that the three mutant proteins were expressed as the wild-type full-length protein when they were expressed from the inducible promoter (data not shown). As can be seen in Fig. 8, none of the three mutant proteins (PolXc, PolX$_{G104VG106V}$ and PolX$_{PHP}$) restored the γ-ray resistance of a Δ*polX* mutant strain. Interestingly, expression of the mutant proteins resulted in a slightly

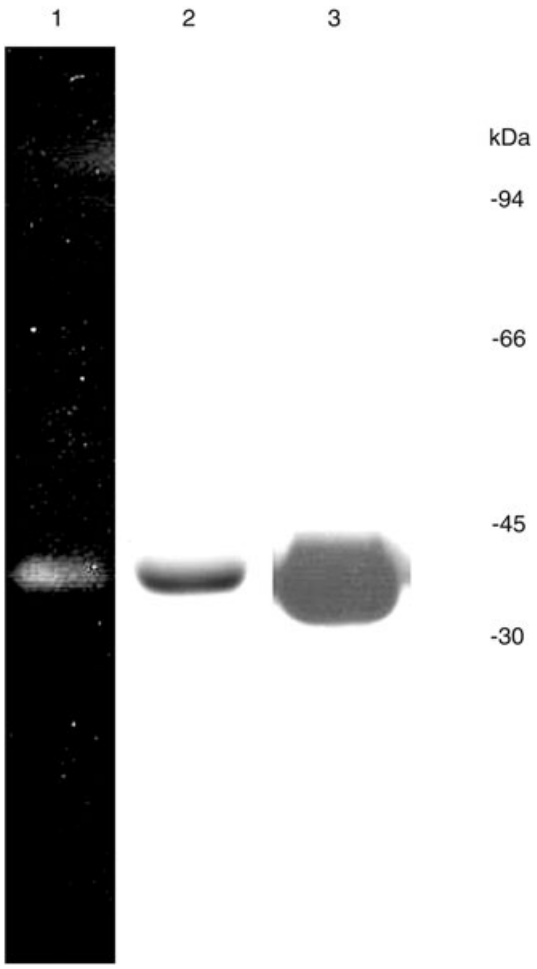

Fig. 6. The PolXc domain harbours the 3′→5′ nuclease activity. The purified PolXc domain of $PolX_{Dr}$ was analysed on a nuclease *in situ* activity gel containing ϕX174-DNA with an annealed [^{32}P]-labelled primer (lane 1) and confirmed by Coomassie staining (lane 2) and Western blot using IgG against the full-length His-$PolX_{Dr}$ (lane 3).

increased γ-ray sensitivity of a Δ*polX* host (Fig. 8). This effect was much more significant with the mutant PolXc.

To investigate further the impact of the $PolX_{Dr}$ 3′→5′ exonuclease activity in the repair of radiation-induced DNA damage, we measured the kinetics of repair of double-stranded DNA breaks in cells exposed to 6800 Gy γ-irradiation. This dose was chosen because it introduces about 200 double-stranded DNA breaks into each genome of a *D. radiodurans* cell (Battista, 1997) but does not affect the viability of wild type or *polX* mutant strains (Fig. 8). We also measured the kinetics of recovery of exponential growth after irradiation. As compared with wild-type *D. radiodurans*, cells devoid of $PolX_{Dr}$ had a 30 min delay in the reconstitution of an intact genome (Fig. 9A and B) and this delay was longer (60–90 min) when these cells expressed a mutant $PolX_{Dr}$ protein (Fig. 9D, E and F for $PolX_{PHP}$, $PolX_{G104VG106V}$ and PolXc respectively). Consistent with the slow kinetics of mending DSB, the kinetics of recovery of exponential growth after irradiation in cells expressing a mutant $PolX_{Dr}$ protein also showed an increased growth lag as compared with cells devoid of $PolX_{Dr}$ (Fig. 10, circles and crosses). These results suggested that the structure-modulated 3′→5′ exonuclease activity of $PolX_{Dr}$ is important for DSBR.

Discussion

We showed that $PolX_{Dr}$ is not only a DNA polymerase but also a 3′→5′ exonuclease. Gel filtration and nuclease *in situ* activity gels excluded that the nuclease activity results from an *E. coli* contaminant. Another fact that argues against a contamination by an *E. coli* nuclease is that no known nuclease from *E. coli* shows the same biochemical properties like $PolX_{Dr}$ (see also Table 1). Characterization of the nuclease activity showed 3′→5′ exonuclease activity on single- and double-strand DNA. Furthermore, the 3′→5′ exonuclease digests the DNA in a very processive

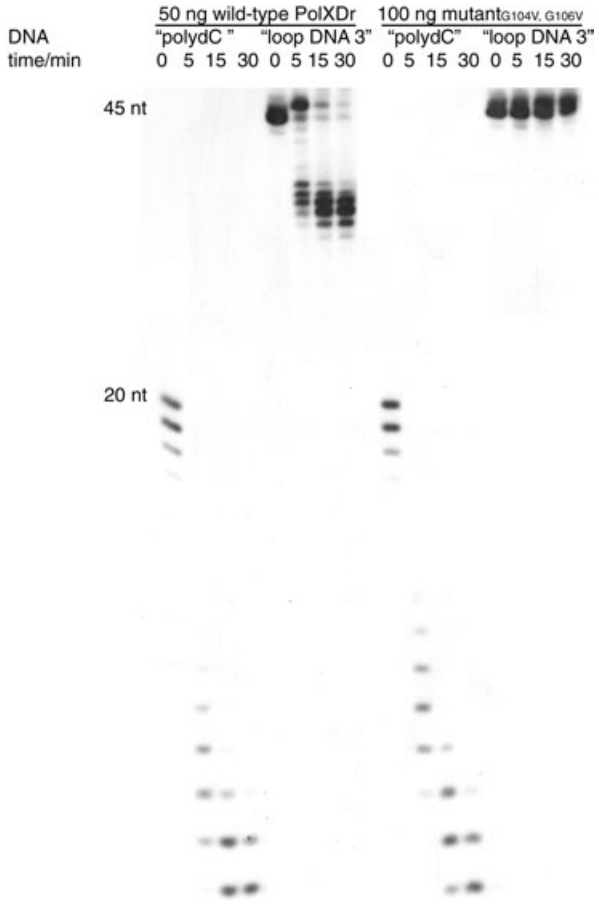

Fig. 7. Mutations of two conserved glycines 104 and 106 lead to loss of the structure-modulated 3′→5′ exonuclease activity. Time-course experiments were done in parallel for the wild type and the mutant enzyme. One hundred nanograms of wild type and 150 ng of mutant $PolX_{Dr}$ were incubated with 25 fmol of 5′-labelled pC 20mer or loop DNA 3 for the indicated time points.

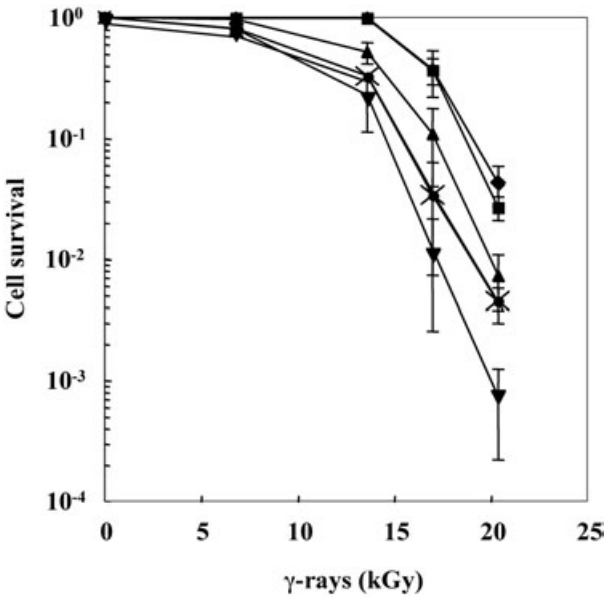

Fig. 8. The Δ*polX*$_{Dr}$, *polX*$_{PHP}$, *polX*$_{G104VG106V}$ and *polX*c bacteria show increased sensitivity to γ-irradiation. Bacteria were exposed to γ-irradiation at doses indicated on the abscissa and the experiments carried out as described in *Experimental procedures*. Values are averages ± standard deviation derived from four independent experiments. Symbols: wild type (squares), Δ*polX*$_{Dr}$/p11549 (triangles), Δ*polX*$_{Dr}$/p11549-*polX*$_{Dr}$ (diamonds), Δ*polX*$_{Dr}$/p11549-*polX*$_{PHP}$ (crosses), Δ*polX*$_{Dr}$/p11549-*polX*$_{G104VG106V}$ (circles), Δ*polX*$_{Dr}$/p11549-*polXc* (inverted triangles).

manner (data not shown). Considering the phenotype of PolX$_{Dr}$ knockout cells (Lecointe *et al*., 2004), we assume that this exonuclease is important for DSBR.

Striking, however, is the fact that PolX$_{Dr}$ is a modulated 3′→5′ exonuclease when it encounters a stem-loop oligonucleotide. So far, rather structure-specific endonucleases than 3′→5′ exonucleases that are affected by DNA structures have been found that specifically recognize and process stem loops, among them the Mre11 complex which was suggested to play a role in DSBR through non-homologous end-joining (NHEJ) (D'Amours and Jackson, 2002).

The DNA polymerases and nucleases involved in NHEJ in eukaryotes have still not been fully identified. It was suggested that the eukaryotic polymerase X family member pol λ and Mre11 participate in this pathway. Recently, NHEJ was also identified in the prokaryotes *B. subtilis* and *M. tuberculosis* (Weller *et al*., 2002). We argue that being a DNA polymerase and a stem loop-modulated 3′→5′ exonuclease, PolX$_{Dr}$ might be involved in DSBR in *D. radiodurans*. This idea is supported by our *in vivo* data showing that deletion of *polX*$_{Dr}$ leads to a significant decrease in radiation tolerance and furthermore that expression of functionally impaired PolX$_{Dr}$ even more decreases *D. radiodurans* ability to survive and repair strand breaks. It might be that a truncated or mutated form of PolX$_{Dr}$ interacts with broken DNA and other repair factors and thereby blocks an alternative repair pathway that can take over when PolX$_{Dr}$ is completely absent in the knockout cells.

Few hypotheses can be proposed for the precise role of PolX$_{Dr}$ in DNA double-strand breaks: (i) the polymerase activity of PolX$_{Dr}$ has been proposed, as have the polymerase activities of human pol λ and *Saccharomyces cerevisiae* Pol4, to play a direct role in an error-prone Ku-independent NHEJ pathway (Wilson and Lieber, 1999; Bebenek *et al*., 2003; Lecointe *et al*., 2004). This pathway uses limited base pairing (microhomologies) between single-strand ends to allow end-joining in order to ensure the repair of radiation-induced DSBs with incompatible or damaged bases that cannot be joined by the Ku-dependent error-free pathway (Moore and Haber, 1996; Ma *et al*., 2003). (ii) The 3′→5′ exonuclease activity of PolX$_{Dr,}$ modulated by stem-loop structures may play some role(s) in DNA repair reminiscent of those of the Mre11 complex, which does not possess any polymerase activity but exhibits both structure-specific endonuclease and 3′→5′ exonuclease activities (Aravind and Koonin, 1998) enhanced for substrates with duplex DNA ends (Trujillo *et al*., 1998; Trujillo and Sung, 2001). Analysis of the sequence of the *D. radiodurans* genome indicates the presence of *sbcD* and *sbcC* genes encoding functional homologues of the proteins of the Mre11 complex (Makarova *et al*., 2001). We propose that PolX$_{Dr}$ and the Deinococcal SbcCD complex may have some redundant functions in DNA DSBR. In particular, they may be involved in processing of DNA ends containing clustered lesions or secondary structures in cells exposed to very high doses of γ-rays. These hypotheses need to be further tested.

This is the first time that a 3′→5′ exonuclease activity was shown to be associated with a member of the polymerase family X. So far a high diversity of enzymatic activities has been found for the different polX family members (Ramadan *et al*., 2004), suggesting that the PolXc domain might be involved in many different processes depending on the protein structure. PolX$_{Dr}$ contains also the PHP domain with unknown function. The fact that, in the absence of the PHP domain, the PolXc domain expressed in Δ*polX*$_{Dr}$ cells had a negative effect on survival of γ-irradiated cells suggests that this domain possesses a function in DNA repair.

The combination of a PolXc domain with a PHP domain is found in many predicted DNA polymerases (Aravind and Koonin, 1998), mainly in archaea, but so far none of them has been characterized. It might therefore well be that these proteins comprise a polymerase activity together with a structure-modulated 3′→5′ exonuclease and are part of a DSBR pathway.

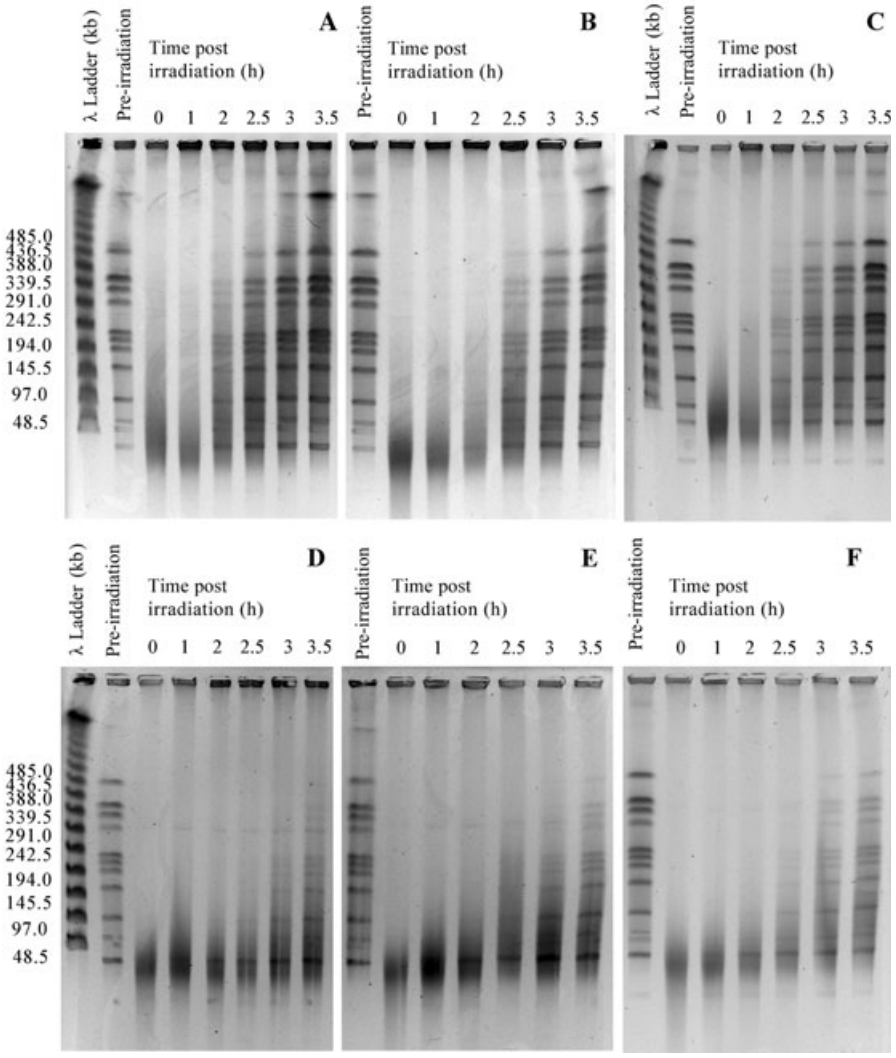

Fig. 9. Kinetics of restoration of genomic DNA. Bacteria were treated as described in Fig. 8. DNA agarose plugs were prepared at the indicated post-irradiation times and digested with *Not*I before analyses by PFGE as described in *Experimental procedures*. A: wild type; B: Δ*polX*$_{Dr}$/p11549; C: Δ*polX*$_{Dr}$/p11549-*polX*$_{Dr}$; D: Δ*polX*$_{Dr}$/p11549-*polX*$_{PHP}$; E: Δ*polX*$_{Dr}$/p11549-*polX*$_{G104VG106V}$ and F: Δ*polX*$_{Dr}$/p11549-*polXc*.

Experimental procedures

Materials

[γ-^{32}P]-ATP, [α-^{32}P]-dCTP and [^{3}H]-dCTP as well as all chromatographic columns were from Amersham Pharmacia Biotech. Oligonucleotides were obtained from Microsynth (Balgach, Switzerland). The M13mp2 DNA was prepared following a standard protocol (Kunkel, 1985). The pMALc2e vector, the ϕX174 DNA, all DNA modifying enzymes and restriction endonucleases were from New England Biolabs. MC1061 cells were obtained from Clontech.

Cloning and expression of PolX$_{Dr}$

PolX$_{Dr}$ was expressed and purified with a His-tag on the NH_2-terminus as previously described (Lecointe *et al*., 2004). To obtain the protein with a N-terminal MBP-tag, the *polX*$_{Dr}$ gene was amplified by polymerase chain reaction (PCR) from the pZE14-*polX*$_{Dr}$ plasmid with the following primers: Dr-M-F 5′CGGGGTACCGACCCTGCCGCCCGACG3′ and Dr-M-R 5′CCCCAAGCTTTATGCACGGTCCGCCGG3′, and cloned between the restriction sites for KpnI and HindIII (underlined sequences) of a pMALc2e vector. The resulting plasmid was then transformed into MC1061 cells and expression was induced with 0.3 mM IPTG at an OD_{600} of 0.25. Expression was carried out for 2 h at 37°C. After centrifugation (4000 rpm, H6000A rotor, 30 min, +4°C) the pellet of 2 l culture was resuspended in 50 ml buffer A (20 mM Tris/HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF, 1 μM benzamidine, 5 μg ml^{-1} leupeptin and 2 μg ml^{-1} pepstatin A). The cells were lysed by French press and sonicated on ice for 2 min. After centrifugation (20 000 rpm, SS34 rotor, 30 min, +4°C) the soluble cell extract was loaded on a hand-made amylose column pre-equilibrated with buffer A. The bound protein was eluted with 10 mM maltose in buffer A. After desalting to buffer B [40 mM Tris/HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 15% (v/v) glycerol and protease inhibitors, as above], the eluate was loaded onto a 1 ml HiTrap® heparin column and proteins were eluted with a NaCl-gradient from 50 to 1000 mM. PolX$_{Dr}$ eluted at 400 mM NaCl. The peak

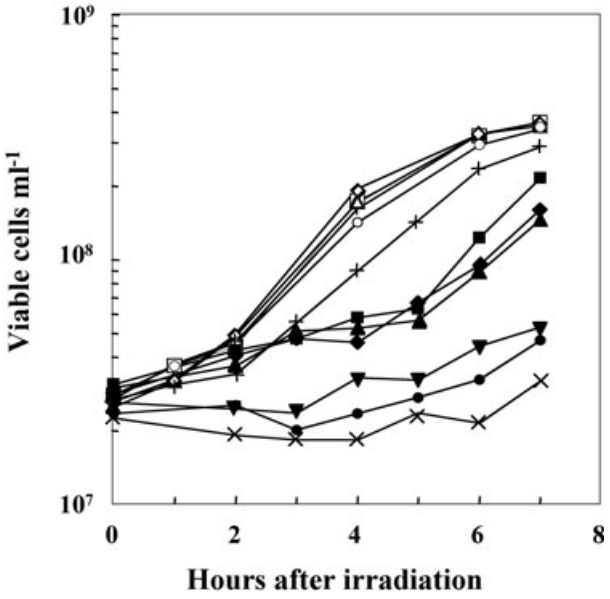

Fig. 10. The Δ*polX*$_{Dr}$ bacteria show an increased delay in cell division and in intact genomic DNA restoration. Bacteria, wild type (squares), Δ*polX*$_{Dr}$/p11549 (triangles), Δ*polX*$_{Dr}$/p11549-*polX*$_{Dr}$ (diamonds), Δ*polX*$_{Dr}$/p11549-*polX*$_{PHP}$ (crosses or plus symbols), Δ*polX*$_{Dr}$/p11549-*polX*$_{G104VG106V}$ (circles), Δ*polX*$_{Dr}$/p11549-*polX*c (inverted triangles), were exposed (closed symbols or crosses) or not (open symbols or plus symbols) to γ-irradiation at a dose of 6800 Gy, diluted in TGY2X to an OD_{650} = 0.2 and incubated at 30°C as described in *Experimental procedures*. At different times after irradiation, aliquots were taken to measure the number of viable cells per ml.

fractions were pooled, desalted to buffer B and loaded onto a Mono Q column. The protein was eluted at 320 mM NaCl in buffer B. The yield from 2 l of culture was 150 μg MBP-PolX$_{Dr}$ with a purity of > 90%.

We also expressed and purified a truncated form of PolX$_{Dr}$ containing the first 313 amino acids including the PolXc domain. For this we amplified the corresponding DNA fragment by PCR with the following primers: DrNheF 5′CCGCGCTAGCACCCTGCCGCCCGACGC3′ and Dr313R 5′CCGCGATATC*TTA*TTGCCAGAGGTCGTCGTGC3′ introducing restriction sites for NheI and EcoRV respectively (underlined sequences) as well as a stop codon (in italics). The obtained fragment was then ligated into a pZE14-vector (Lutz and Bujard, 1997) and the protein was expressed and purified exactly like the full-length protein. As discussed in the *Results*, the protein yield was strongly decreased compared with the full-length protein. In order to express the PHP domain of PolX$_{Dr}$ consisting of the amino acids 301–573. The DNA fragment was amplified by PCR using the following primers: HisP-F 5′CCGCGCTAGCGCCGAGTACCGCGAG3′ and HisP-R 5′CCGCGATATC*TTA*TGCACGGTCCGCCGG3′ introducing restriction sites for NheI and EcoRV respectively (underlined sequences) and a stop codon (in italics).

Expression of the wild type and mutant PolX$_{Dr}$ *proteins from a P*spac *promoter in* D. radiodurans

Expression of PolX$_{Dr}$ in *D. radiodurans* was performed by transforming Δ*polX*$_{Dr}$ bacteria by a plasmid that expresses *polX*$_{Dr}$ gene from an inducible promoter. For this, the *polX*$_{Dr}$ gene was cloned into the shuttle *E. coli–D. radiodurans* vector p11549 (Lecointe *et al*., 2004) that contains the IPTG-inducible P*spac* promoter and the cognate *lacI* regulatory gene. Plasmid p11549-*polX*$_{Dr}$ was constructed by replacing the NdeI–XhoI portion of the vector p11549 downstream of the P*spac* promoter with the 1892 bp NdeI–XhoI fragment corresponding to the coding region of the *polX*$_{Dr}$ gene. This fragment was obtained by PCR amplification using *D. radiodurans* genomic DNA as template and primers 5′GGAACATATGACCCTGCCGCCCGACGC3′ and 5′GGAA CTCGAGTTATGCACGGTCCGCCGGGC3′ tagged with restriction sites NdeI and XhoI (underlined) respectively.

Generation of a mutant G104V,G106V PolX$_{Dr}$

Mutagenic PCR was performed using TurboPfu™(Stratagene) and the following primers: mutGG-F 5′GGCGTG CGCGTGCTGGTGCCGAAGAAGATTCG3′ and mutGG-R 5′CGAATCTTCTTCGGCACCAGCACGCGCACGCC3′. The parental non-mutated pZE14 vector template was digested with DpnI (New England Biolabs) and the mutated plasmid was transformed into *E. coli* and plasmid DNA was isolated. Introduction of the desired mutation was confirmed by DNA sequencing. Protein expression and purification was carried out exactly like for the wild-type protein.

Gel filtration

For gel filtration a Superose 12™ column was used following the supplied instruction. The gel filtration buffer contained 40 mM Tris/HCl pH 7.5, 15% (v/v) glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.5 M NaCl. 250 μl (containing 34 μg) of His-PolX$_{Dr}$ was loaded. The flow rate was 0.5 ml min^{-1} and fraction size was 0.5 ml. Fractions were stored at –20°C before analysis.

Nuclease assays

The general nuclease activity was determined using activated DNA that was prepared following a standard protocol (Wang *et al*., 1999) and that was labelled by performing a gap-filling reaction with Klenow fragment exo$^-$ and [^{3}H]-dNTPs. Nuclease reactions of 15 μl were performed in reaction buffer X (40 mM Tris/HCl pH 8, 1 mM 2-mercaptoethanol and 1 mM $MnCl_2$) for 30 min at 37°C unless indicated otherwise. The reactions were stopped by addition of 20 μl stop solution (50 mM EDTA, 5 mg ml^{-1} BSA) and undigested DNA was precipitated with 40 μl of 25% trichloroacetic acid for 10 min on ice. After 15 min centrifugation with 15 300 *g*, the percentage of released radioactivity in the supernatant was measured by bioscintillation counting and represented as per cent of the total radioactivity per reaction.

Nuclease in situ *activity gel*

The DNA substrate was ϕX174 DNA with an annealed 15mer primer 1 (for sequence see Table 2) that was elongated using

[α-^{32}P] dCTP and Klenow fragment exo$^-$. DNA was added to a SDS gel mix (10% polyacrylamide) directly before polymerization. The proteins were heated for 2 min at 70°C before loading. Protein separation and *in situ* renaturing of the enzymes was performed like previously described (Maga *et al*., 2002). After renaturing, the nuclease activity was detected by incubating the gel 12 h at room temperature with 0.01% Triton X-100 under the conditions described for the nuclease assay. Molecular mass markers were visualized with Coomassie brilliant blue R250 and transferred onto the autoradiograph.

Product analysis assay

Unless otherwise indicated, all DNA substrates were prepared as follows: an oligonucleotide was labelled at the 5′ end using polynucleotide kinase and [γ-^{32}P]-ATP, annealed to unlabelled oligonucleotides when required and purified on a MicroSpin™ G-25 column (Amersham Pharmacia Biotech). All nuclease assays were carried out in a total volume of 10 µl in reaction buffer X with 25 fmol of DNA substrate per reaction. The reactions were started upon addition of PolX$_{Dr}$ and incubated for 30 min at 37°C unless indicated otherwise. The reactions were stopped by addition of 6 µl of loading buffer (96% (v/v) formamide, 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). The samples were heated for 3 min at 98°C and then directly loaded on a denaturing polyacrylamide gel [15–18% polyacrylamide with 7–8 M urea and 15% (v/v) of formamide]. For optimal separation, we used a Sequi-Gen™ sequencing gel (Bio-Rad) in TBE buffer at 50°C and the DNA was visualized by autoradiography.

Media and cultures

Cultures of *D. radiodurans* and *E. coli*, media, and transformation of *D. radiodurans* with plasmid or genomic DNA were done as described by Bonacossa de Almeida *et al*. (2002). When necessary, media were supplemented with the appropriate antibiotics used at the following final concentrations: ampicillin, 100 µg ml^{-1} for *E. coli*; chloramphenicol, 20 µg ml^{-1} for *E. coli* or 3 µg ml^{-1} for *D. radiodurans*; kanamycin, 6 µg ml^{-1} for *D. radiodurans*; spectinomycin, 40 µg ml^{-1} for *E. coli* or 75 µg ml^{-1} for *D. radiodurans*.

γ-Irradiation

Bacteria containing plasmid p11549 or derivatives expressing wild type or mutant PolX$_{Dr}$ proteins were grown in TGY2X supplemented with 10 mM IPTG and chloramphenicol to an OD_{650} = 2–3. The cultures were concentrated 10 times in TGY2X and irradiated on ice with a ^{137}Cs irradiation system (Institut Curie) at a dose rate of 56.6 Gy min^{-1}. Following irradiation, diluted samples were plated on TGY plates supplemented with 1 mM IPTG and incubated for 3–5 days at 30°C before the colonies were counted.

Kinetics of DNA DSBR by pulsed field gel electrophoresis

Cells were exposed to 6800 Gy γ-irradiation. Irradiated cultures and unirradiated control were diluted to an OD_{650} of 0.2 in TGY2X supplemented with 10 mM IPTG and incubated at 30°C. At different post-irradiation incubation times, culture aliquots (5 ml) were taken to prepare DNA plugs as described by Harris *et al*. (2004). The DNA in the plugs was digested for 16 h at 37°C with 10 units of NotI restriction enzyme. After digestion, the plugs were subjected to pulsed field gel electrophoresis (PFGE) for 22 h at 12°C using a CHEF MAPPER electrophoresis system (Bio-Rad) with the following conditions: 6.0 V cm^{-1}, linear pulse ramp of 10–60 s, and a switching angle of 120° (–60° to +60°). Recovery from DNA damage was monitored by the appearance of the complete pattern of the 11 resolvable fragments generated by NotI digestion of total genomic DNA (Kikuchi *et al*., 1999). In parallel, the aliquots were diluted and plated on TGY agar supplemented or not with 1 mM IPTG; colonies were counted after 3- to 4-day incubation at 30°C to estimate survival in liquid medium and re-initiation of cell division during post-irradiation incubation.

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6.2 Enzymes involved in DNA ligation and end-healing in the radioresistant bacterium *Deinococcus radiodurans*

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Enzymes involved in DNA ligation and end-healing in the radioresistant bacterium *Deinococcus radiodurans*

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Abstract

Background

Enzymes involved in DNA metabolic events of the highly radioresistant bacterium *Deinococcus radiodurans* are currently examined to understand the mechanisms that protect and repair the *Deinococcus radiodurans* genome after extremely high doses of γ-irradiation. Although several *Deinococcus radiodurans* DNA repair enzymes have been characterised, no biochemical data is available for DNA ligation and DNA end-healing enzymes of *Deinococcus radiodurans* so far. DNA ligases are necessary to seal broken DNA backbones during replication, repair and recombination. In addition, ionizing radiation frequently leaves DNA strand-breaks that are not feasible for ligation and thus require end-healing by a 5'-polynucleotide kinase or a 3'-phosphatase. We expect that DNA ligases and end-processing enzymes play an important role in *Deinococcus radiodurans* DNA strand-break repair.

Results

In this report, we describe the cloning and expression of a *Deinococcus radiodurans* DNA ligase in *Escherichia coli*. This enzyme efficiently catalyses DNA ligation in the presence of Mn(II) and NAD^+ as cofactors and lysine 128 was found to be essential for its activity. We have also analysed a predicted second DNA ligase from *Deinococcus radiodurans* that is part of a putative DNA repair operon and shows sequence similarity to known ATP-dependent DNA ligases. We show that this enzyme possesses an adenylyltransferase activity using ATP, but is not functional as a DNA ligase by itself. Furthermore, we identified a 5'-polynucleotide kinase similar to human polynucleotide kinase that probably prepares DNA termini for subsequent ligation.

Conclusions

Deinococcus radiodurans contains a standard bacterial DNA ligase that uses NAD^+ as a cofactor. Its enzymatic properties are similar to *E. coli* DNA ligase except for its preference for Mn(II) as a metal cofactor. The function of a putative second DNA ligase remains unclear, but its adenylyltransferase activity classifies it as a member of the nucleotidyltransferase family. Characterization of another protein from the same operon revealed a 5'-polynucleotide kinase with a possible role in DNA strand-break repair.

Background

Deinococcus radiodurans

Deinococcus radiodurans (D. radiodurans) exhibits an extraordinary resistance to ionizing radiation. Ionizing radiation generates a variety of DNA damages, including many types of base damages as well as single-strand and double-strand breaks, the latter being the most lethal damage for a living cell. *D. radiodurans* can survive irradiation up to 5,000 Gy without measurable loss of viability, and it seems likely that this resistance is based on mechanisms that ensure limited DNA and protein degradation and provide an efficient and accurate DNA strand-break repair [1]. High intracellular levels of Mn(II) protect proteins and allow fast repair of damaged DNA after irradiation [2, 3]. Prokaryotes can repair double-strand breaks by homologous

recombination, but proteins implicated in non-homologous end-joining have also been identified recently, such as Ku homologs and additional DNA ligases [4, 5]. However, no Ku homolog has been discovered in the genome of *D. radiodurans*. Zahradka *et al*. found that a mechanism called extended synthesis-dependent strand-annealing accounts for most of the strand-break repair [6], although additional DNA repair pathways might contribute to the efficient DNA repair. In any case, a DNA ligase is essential for DNA repair and a 5'-polynucleotide kinase/3'-phosphatase would ensure that DNA strand-breaks could be invariably ligated.

DNA ligases

DNA ligases play essential roles in replication, recombination and repair since they join broken DNA strands by catalysing the formation of a phosphodiester bond between the 3' hydroxyl end of one strand and the 5' phosphate end of another. Ligation occurs via three nucleotidyltransfer steps: (i) a covalent enzyme-adenylate intermediate is formed, (ii) the adenylate group (AMP) is transferred to the 5'-phosphate terminus of the DNA molecule and (iii) the gap in the DNA molecule is sealed when the DNA ligase catalyses displacement of the AMP residue through the attack by the adjacent 3' hydroxyl group of the DNA [7]. For all DNA ligases, the AMP is linked to a highly conserved lysine residue in the catalytic motif of the enzyme. DNA ligases can use either ATP or NAD^+ as an AMP-donor. NAD^+-dependent DNA ligases are found exclusively in bacteria, certain archaea, and viruses whereas ATP-dependent DNA ligases can be found in eukaryotes, archaea and several viruses including bacteriophages. Recently, it was shown that some bacterial genomes also encode an additional ATP-dependent DNA ligase, some of which were further characterised [7].

The *D. radiodurans* genome contains the gene DR2069 encoding an NAD^+-dependent DNA ligase, here designated as LigA. The gene DRB0100 encodes another possible diverged homolog of ATP-dependent ligases. As the function of this protein remains unclear it will be called DRB0100 throughout this paper. This predicted ATP-dependent DNA ligase contains all catalytic residues, and its expression is strongly upregulated upon γ-irradiation [8]. In addition, DRB0100 belongs to a putative DNA repair operon together with the genes DRB0098 and DRB0099. DRB0098 has been predicted to encode a kinase/phosphatase with an unusual domain architecture [9] whereas DRB0099 is classified as a domain of unknown function with weak similarity to the macro domain family [10].

Polynucleotide kinases and 3' phosphatases

Not all DNA strand breaks possess ligatable ends, i.e. a 5' phosphate and a 3' OH terminus. The 5' phosphate can be missing and γ-irradiation and reactive oxygen can lead to the formation of 3' phosphate or phosphoglycolate ends [11, 12]. Enzymatic activity is required to remove the 3' phosphate moiety and to phosphorylate the 5' end at the DNA nick to allow for DNA ligation. Both reactions are catalysed by bifunctional PNKPs. The best characterised PNKP is T4 PNK that is involved in the repair of host tRNA [13]. Additional PNKPs were identified in other viruses and all these viral enzymes can use either DNA or RNA as a substrate. PNKPs were also found in some eukaryotes, e.g. human, *Caenorhabditis elegans* and *Schizosaccharomyces pombe*, where they seem to play an important role in the repair of single-strand and double-strand breaks [14-16]. However, the eukaryotic enzymes can only use DNA as a substrate. Pnk1 from *Schizosaccharomyces pombe* possesses both 3'-phosphatase and 5'-polynucleotide kinase activities, whereas TPP1 from

Saccharomyces cerevisiae shows only 3'-phosphatase activity. In other organisms, the kinase and phosphatase activities seem to be uncoupled as well, e.g. in *Arabidopsis thaliana*. Only one bacterial PNKP from *Clostridium thermocellum* has been characterised so far [17], showing similarity to viral PNKPs. *D. radiodurans* also seems to possess a PNKP encoded by the gene DRB0098, although the PNKP possesses a special domain architecture [9]. The order of the phosphatase and kinase domains is the similar to eukaryotic PNKPs; in contrast, viral PNKPs have a reversed order of the two domains. The predicted phosphatase domain of the *D. radiodurans* PNKP belongs to the HD hydrolase superfamily [18], and, so far, only one viral PNKP containing this domain has been shown to possess 3'-phosphatase activity [19]. The *D. radiodurans* PNKP is part of the putative DNA repair operon together with the predicted ATP-dependent DNA ligase DRB0100 and the expression of this operon is strongly upregulated upon irradiation. Thus, a role for the encoded proteins in DNA repair has been suggested [8].
In this work we analyse two putative DNA ligases and one predicted 5'-polynucleotide kinase/3'-phosphatase from *Deinococcus radiodurans*.

Results

Prediction of two DNA ligases for *D. radiodurans*

Sequence comparison of the two predicted *D. radiodurans* DNA ligases with other bacterial DNA ligases showed that LigA displays a strong similarity to other NAD^+-dependent DNA ligases (Figure 1A) and comprises the expected adenylation, OB fold and BRCT domains. Like other NAD^+-dependent DNA ligases LigA also contains a zinc finger and a helix-hairpin-helix motif presumably involved in DNA binding (Figure 1B). By contrast, the predicted ATP-dependent DNA ligase DRB0100 shows poor sequence similarity to other bacterial ATP-dependent ligases, but contains all catalytic residues (Figure 1A and [8]). The DRB0100 protein consists of the adenylation domain only and lacks all other domains present in LigA (Figure 1B); especially no DNA binding motif could be detected.

Purification of two recombinant DNA ligases from *D. radiodurans*

Both genes encoding putative DNA ligases, DRB0100 and DR2069, were amplified from genomic *D. radiodurans* DNA using specific primers (see Table 1) and cloned into a pRSETb vector for recombinant protein expression in *E. coli* cells with a hexa-histidine tag at the N terminus. For both proteins, adenylation mutants were created by replacing the conserved lysine residue with an alanine, resulting in a DRB0100 K40A mutant and a LigA K128A mutant, respectively. All wild-type and mutant proteins were expressed in *E. coli* BL21(DE3) cells and purified to near homogeneity over a HisTrap™ HP column and two additional ion exchange columns (Figure 1C).

A DNA ligase from *D. radiodurans* performs efficient strand joining in the presence of NAD^+ and Mn(II) and possesses adenylyltransferase activity

We tested the ability of the LigA wt and the K128A mutant to ligate a duplex DNA substrate containing a single nick. Ligase activity was measured as conversion of a 5'-[^{32}P]-labelled deoxyribose oligomer of 19 nucleotides into an internally labelled oligomer of 44 nucleotides. LigA showed maximum ligation activity with 1 mM $MnCl_2$, 5 µM NAD^+ and a pH of 6.8 at a temperature of 30°C. Higher concentrations of $MnCl_2$ or NAD^+ had an inhibitory effect on the enzymatic activity. The enzyme was 10 times less active in the presence of $MgCl_2$, and even inactive when tested with

1 mM ATP (data not shown). To exclude the possibility that the observed activity is caused by a copurified *E. coli* ligase, we created a K128A mutant that lacks the proposed site of adenylation (Figure 1A). The LigA K128A mutant showed almost no ligation activity confirming that the observed ligation activity results from the *D. radiodurans* NAD^+-dependent DNA ligase (Figure 2A). The residual DNA ligation does probably not result from a contamination with *E. coli* DNA ligase, as the activity was strongly decreased in presence of 4 mM $MgCl_2$, which is optimal for *E. coli* DNA ligase (data not shown). In an adenylyltransferase activity assay LigA wt formed an AMP-ligase complex, whereas complex formation was not detected with the K128A mutant (Figure 2A, right). Thus, lysine 128 is essential for the first step of DNA ligation. The kinetic analysis of the wt reaction using different concentrations of nicked DNA displayed typical Michaelis-Menten kinetics with an apparent K_M of 105 ± 16 nM (Table 2).

Divalent cation dependence and specificity of the DNA strand-joining by LigA

Ligation of a nicked DNA by LigA required a divalent cation cofactor and was best in the presence of 1 mM $MnCl_2$ (Figure 3A). $MnCl_2$ could be replaced by $MgCl_2$ or $CaCl_2$ leading however to a 10-fold decrease of activity (Figure 3A). The optimal concentration of divalent cation was 1 mM for $MgCl_2$ and 2 mM for $CaCl_2$. Only low levels of DNA ligation were observed with $NiCl_2$ and $ZnCl_2$, the optimal concentrations being 2 and 3 mM, respectively (Figure 3B). $CoCl_2$ could not serve as a divalent cation cofactor (Figure 3B).

DRB0100, a predicted ATP-dependent DNA ligase from *D. radiodurans*, forms a complex with AMP, but does not ligate DNA or RNA *in vitro*

DRB0100 has been predicted to be an ATP-dependent DNA ligase consisting only of the adenylation domain. We first tested whether the DRB0100 protein possesses an adenylation activity using ATP as an AMP-donor and whether lysine residue 40 is indeed essential for AMP binding. The adenylyltransferase activity was tested by incubating 1 μg of recombinant protein with α-[^{32}P]-ATP. A complex was formed between the wild-type protein and [^{32}P]-AMP, which was completely absent for the K40A mutant, confirming that DRB0100 possesses adenylyltransferase activity and therefore belongs to the family of nucleotidyltransferases (Figure 2B). We further tested whether DRB0100 is able to ligate DNA or RNA substrates using NAD^+ or ATP as a cofactor. However, we did not detect a ligation product with any conditions used [see Additional file 1 and Additional file 2].

Purification of a putative 5'-polynucleotide kinase/3'-phosphatase from *D. radiodurans* with an unusual domain architecture

The PNKP encoded by *D. radiodurans* has a phosphatase-kinase domain architecture similar to the eukaryotic PNKPs. In contrast, the viral T4 PNK has a reverse domain order with the kinase domain at the N-terminus and the phosphatase domain at the C-terminus. Comparison of *D. radiodurans* and human PNKP shows that the bacterial protein is smaller than the human homolog and contains a phosphatase domain belonging to the superfamily of HD phosphohydrolases. The human enzyme contains a distinct phosphatase domain with some similarity to histidinol phosphatase and related phosphatases (Figure 4A).

The gene DRB0098 encoding a putative PNKP was amplified via PCR from genomic *D. radiodurans* DNA. The gene was cloned into a pRSETb vector and arginine 371 was mutated to lysine using mutagenic primers for PCR. Arginine 371 was chosen based on sequence comparisons with the well-characterised T4 PNK. We estimated that it should correspond to arginine 126 in T4 PNK, which is required for polynucleotide kinase activity [20].
Both proteins, DRB0098 wt and DRB0098 R371K were expressed in *E. coli* BL21(DE3) cells with an N-terminal hexa-histidine tag. The proteins were purified over a HisTrap HP™ column, a HiTrap Heparin HP™ column and finally a HiTrap SP HP™ column to apparent homogeneity (Figure 4B).

Analysis of the *D. radiodurans* PNKP polynucleotide kinase/ 3'-phosphatase activity

Polynucleotide activity for the *D. radiodurans* PNKP was shown as transfer of $^{32}P_i$ from γ-[^{32}P]-ATP to the 5'OH end of a 25mer oligodeoxyribonucleotide. The resulting 5'[^{32}P]-labelled product was separated from the free γ-[^{32}P]-ATP by polyacrylamide gel electrophoresis and detected by autoradiography. The wild-type protein showed clear 5'-polynucleotide kinase activity with an optimal $MnCl_2$ concentration of only 0.25 mM. Mutation of arginine 371 to a lysine strongly reduced the enzymatic activity (Figure 4C), confirming that the kinase activity is intrinsic to the C-terminal domain. Furthermore, as *E. coli* does not possess a polynucleotide kinase, a contamination can be excluded.
The 3'-phosphatase activity was analysed as conversion of a non-ligatable DNA nick, which is "blocked" by a 3' PO_4 moiety, to a normal 3'OH-5'PO_4 nick that can be subsequently joined by a DNA ligase. Both, D. *radiodurans* LigA and T4 DNA ligase, were able to ligate the blocked substrate if PNKP was present (data not shown). Even though 3'-phosphatase activity has been detected, we cannot conclude whether this activity is intrinsic to the PNKP or not. Samples purified from *E.coli* cells containing only the empty expression vector contained unspecific 3'-phosphatase activity as well and H81A or D82E mutants of DRB0098 did not show any reduced 3'-phosphatase activity, although these two residues represent the conserved HD motif (data not shown). An enzymatic mutant of DRB0098 is required to definitely decide this open question.

Discussion

DNA ligases are important enzymes acting in DNA replication, recombination and repair. They can be classified by cofactor requirement: those requiring NAD^+ and those requiring ATP [21]. For many years it was believed that bacteria possess only NAD^+-dependent DNA ligases. However, several years ago, it became clear that some bacteria contain an ATP-dependent DNA ligase in addition to their NAD^+-dependent DNA ligase [22]. The presence of these ligases suggested that prokaryotes, similar to eukaryotes, could have specific DNA ligases that act in DNA repair and recombination.
In this work, we report the identification of LigA, an NAD^+-dependent DNA ligase, and a second putative ATP-dependent DNA ligase in the radioresistant bacterium *D. radiodurans*. NAD^+-dependent DNA ligases are highly conserved and it is likely that they are essential for all bacteria [7]. *D. radiodurans* LigA showed strong ligation activity on a nicked DNA substrate in the presence of NAD^+ and $MnCl_2$, but only a weak activity in the presence of $MgCl_2$. This Mn^{2+} preference is not surprising since it

was shown that these ions are present in extremely high levels at the *D. radiodurans* DNA [23] and are essential for γ-radiation resistance [3]. Moreover, several DNA repair enzymes from *D. radiodurans*, such as UV endonuclease β [24] or a family X DNA polymerase with a structure-modulated nuclease activity [25, 26], are strongly stimulated by $MnCl_2$. The first step in the ligation process is the formation of an adenylated ligase. According to sequence alignment with other NAD^+-dependent DNA ligases, adenylation of the LigA protein is predicted to occur on lysine 128. Indeed, a mutation of this lysine residue to alanine abolished the ligation as well the adenylation activity.

The product of the *D. radiodurans* gene DRB0100, a diverged homolog of ATP-dependent DNA ligases, contains most of the conserved amino acid residues characteristic of DNA ligases and was shown to be strongly upregulated upon γ-irradation [8]. We could show that this protein possesses adenylyltransferase activity using α- [^{32}P]-ATP as a substrate and that the adenylation occurs specifically at the conserved lysine 40. This transfer of radioactivity to the wild-type enzyme, but not to the K40A mutant, indicates a covalent modification of the respective lysine residue as observed for other ligases. This places DRB0100 in the family of nucleotidyltransferases that includes DNA and RNA ligases as well as RNA capping enzymes. As RNA capping is not characterised for prokaryotes, we focussed our work on the possible ligation activity. However, to our knowledge the presence of RNA capping has not been investigated in *D. radiodurans* and can therefore not completely be excluded. Although different cofactors and various buffer conditions as well as different substrates were used, and the hexa-histidine tag was transferred from the N- to the C-terminus of the protein, we were not able to show that DRB0100 is active as a DNA or RNA ligase. Nicked DNA substrates, nicked DNA-RNA hybrids prepared by annealing of a 5' PO_4 and a 3' OH RNA strand to a template DNA strand, single-stranded RNA and double-stranded DNA with blunt-ends or overhangs were tested (data not shown). In addition, we analysed total *D. radiodurans* extract with or without previous γ-irradiation for DNA ligation activity; however no ATP-dependent ligation activity was detectable, even though NAD^+-dependent DNA ligation could be easily detected (data not shown). DRB0100 does not contain any conventional DNA binding motif, suggesting that an additional protein is required for recruitment to nicked DNA.

As DRB0100 is part of a putative repair operon DRB0098-DRB0100, we purified the other two proteins to analyse whether the three operon proteins would form a complex capable of DNA ligation. DRB0098 contains a HD-hydrolase family phosphatase domain and a polynucleotide kinase domain and resembles the human repair protein PNKP [27]; DRB0099 is an open reading frame with unknown function and weak similarity to macro domains [8, 10]. No DNA ligation was detected with any of these three operon proteins or in combinations thereof; thus, we propose the existence of a yet unidentified additional protein involved in the ligation process of DRB0100. Moreover, it cannot be excluded that DRB0100 ligates only special substrates such as specific DNA sequences or RNA intermediates. Interestingly, in several bacteria genes coding for an ATP-dependent DNA ligase have been identified in operons with Ku-homologs. The Ku proteins might recruit the DNA ligase to DNA strand-breaks as is it the case in mammalian cells [28]. In *D. radiodurans,* however, no Ku-homolog has been identified so far. Another interesting protein that might function in a Ku-like manner is the repair protein PprA from *D. radiodurans*, which has been shown to tether DNA ends and to stimulate ATP- and NAD^+-dependent DNA ligases [29, 30].

The ATP-dependent DNA ligase might function as a backup system to provide additional ligation activity under conditions of high genotoxic stress.
In this work, we furthermore characterised a novel PNKP from *D. radiodurans*, which phosphorylates 5' OH termini. It remains unclear whether it is also able to remove 3' phosphate groups, thus converting "blocked" DNA nicks to ligatable ones.
PNKPs can be divided into two subgroups according to their domain architecture: the T4-like kinase-phosphatase proteins found in viruses with a function in RNA repair, and the eukaryal-type phosphatase-kinase group involved in DNA repair. The PNKP from *D. radiodurans* possesses a domain architecture that corresponds to the eukaryal type. So far, only one bacterial PNKP from *Chlostridium thermocellum* has been described, which in contrast to the *D. radiodurans* PNKP contains a calcineurin-type phosphatase domain. This enzyme has been shown to possess 5'-polynucleotide kinase, 2'3'-phosphatase and adenylyltransferase activity and has been implicated in RNA repair [17]. It remains to be elucidated if *D.radiodurans* PNKP is involved in DNA or RNA repair.
The *D.radiodurans* PNKP possesses an N-terminal phosphatase domain belonging to the HD superfamily. Members of this family are known or predicted phosphohydrolases [18], and a novel subfamily of PNKPs consisting of a 5'-kinase and a 3'-HD phosphohydrolase domain has been proposed based on sequence similarities [8, 9, 19]. These enzymes have a conserved doublet of HD residues that is likely to be required for enzymatic activity. So far, only one PNKP has been shown to possess a 3'-phosphatase activity residing in the HD domain, but no mutational analysis is available for this enzyme from the bacteriophage RM378 [19]. However, it was shown, that site-directed mutagenesis of the conserved histidine in a cGMP-phosphodiesterase clearly reduced its catalytic activity [31]. We could show that *D. radiodurans* PNKP possesses 5'-polynucleotide kinase activity. However, the 3'-phosphatase activity detected in our assay might result from an unspecific *E. coli* 3'-phosphatase. H81A or D82E mutants of *D.radiodurans* PNKP did not show a reduced activity in our 3'-phosphatase assays (data not shown). Regarding the polynucleotide kinase activity, the absence of a 5'-polynucleotide kinase in *E.coli* and the reduced activity of the DRB0098 R371K mutant exclude the possibility of a contamination.
In the case of the third protein of the putative repair operon, DRB0099, binding to ADP-ribose was detected and further work has to be done to elucidate whether ADP-ribosylation might play a role in bacterial DNA repair (Blasius, M., and Hübscher, U., unpublished observation).

Conclusions

D. radiodurans possesses a classical NAD^+-dependent DNA ligase (LigA) that shows a strong preference for Mn(II) as a cofactor. A second predicted ATP-dependent DNA ligase (DRB0100) shows adenylyltransferase activity, but no DNA or RNA ligation could be detected *in vitro*. A predicted 5'-polynucleotide kinase/3'-phosphatase belonging to the same operon was able to convert 5' OH termini to 5' PO_4 termini, thus preparing DNA ends for ligation. In conclusion, *D. radiodurans* PNKP and LigA are able to heal and ligate DNA nicks. It remains to be assessed whether they play any role in DNA repair or RNA repair *in vivo*. Also the function of DRB0100 remains to be elucidated and further proteomic and genomic approaches might give more insight into these unsolved questions.

Methods

Bacterial strains and media

E. coli DH5α cells were used for cloning and plasmid preparation (Invitrogen). Recombinant proteins were produced in *E. coli* BL21(DE3) (Novagen). *E. coli* cells were grown in LB medium supplemented with 100 µg/ml ampicillin where required.

Enzymes and reagents

Oligonucleotides synthesis and DNA sequencing were performed by Microsynth. DNA fragments and plasmids were purified with kits from Qiagen. All chemicals used were purchased from Sigma-Aldrich. Immunoblots during protein purifications were done using Tetra-His antibody (Qiagen).

Molecular cloning

Genomic DNA was isolated from *D. radiodurans* R1 type strain as described previously [32] and used as a template for PCR amplification of the different genes. PCR reaction mixtures (50 µl) contained 1X HF buffer (Finnzymes), 200 µM of each dNTP, 400 nM of each forward and reverse primer, 3% DMSO and 2 units of Phusion™ High-Fidelity DNA Polymerase (Finnzymes). Cycling protocols were designed according to the supplier's recommendations and annealing temperatures were determined using the Tm calculator provided by Finnzymes. PCR products were digested with BamHI and ligated into the pRSETb vector (Invitrogen) using T4 DNA ligase (Fermentas). For site-directed mutageneses the plasmid containing the corresponding wild-type gene was used as a template, the annealing temperature was set to 55°C, cycle number was reduced to 12-16, and the PCR product was digested with DpnI to remove the template plasmid. The mutated PCR product was then transformed into DH5α cells, plasmids were isolated and all constructs were verified by sequencing. PCR primer sequences can be found in Table 1.

Expression and purification of recombinant proteins

Cultures of *E. coli* BL21(DE3) cells transformed with the respective expression plasmid were grown in LB medium supplemented with ampicillin at 37°C to an OD_{600nm} of 0.4-0.8, then IPTG was added to 1 mM final concentration and cells were further incubated for 2-4 h at 37°C. Cells were pelleted by centrifugation (4°C, 4,700 g, 30 minutes) in a Sorvall H6000A rotor. All protein purification steps were performed at 4°C or on ice. Cell pellets were resuspended in 30 ml of buffer N (500 mM NaCl, 30 mM phosphate buffer, pH 7.5, 10 mM Tris-HCl, pH 7.5, 10 mM imidazole, and 1 mM PMSF) and lysed with a French press. To ensure complete lysis, cells were in addition sonicated (2 minutes, 40% duty cycle, Branson Sonifier® Cell disruptor B15). The lysate was centrifuged (4°C, 43,000 g, 30 minutes) in a Sorvall SS-34 rotor and the supernatant was loaded onto a 1 ml HisTrap HP™ column (GE Healthcare) using an ÄKTApurifier™ (GE Healthcare). The column was washed with buffer N containing 50 mM NaCl and 50 mM imidazole and protein was eluted with 50 mM NaCl and 300 mM imidazole. Protein was pooled according to a Coomassie Blue R250 stained SDS-PAGE and loaded onto the next column equilibrated in buffer A (40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 15% (v/v) glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol). For LigA wt and K128A mutant the protein was loaded onto a 1 ml Heparin HP™ column and eluted with a gradient from 50 to 1000 mM NaCl in buffer A. LigA eluted at 350 mM NaCl. Protein was pooled, diluted to 50 mM NaCl

with buffer A without NaCl and loaded onto a HiTrap Q HP™ column. Elution was done with a gradient from 50-1000 mM NaCl. Protein eluted at 400 mM NaCl. Fractions that contained nearly homogenous LigA protein as judged by SDS-PAGE were pooled, dialysed to buffer S (20 mM Tris-HCl, pH 7.5, 25% (v/v) glycerol, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 0.5 mM EDTA), and stored at -80°C.

For DRB0100 wt and K40A mutant the pool obtained from the HisTrap HP™ column was loaded onto a 1 ml HiTrap SP HP™ column equilibrated with buffer A. DRB0100 was retrieved in the flow-through, which was diluted to 25 mM NaCl and loaded onto a HiTrap Q HP™ column. Elution was done using a gradient from 25-1000 mM NaCl and DRB0100 protein eluted at 50 mM NaCl. The protein pool was dialysed to buffer S and stored at -80°C.
The HisTrap HP™ pools of DRB0098 wt and R371K mutant were loaded onto a 1 ml Heparin column and eluted as described for LigA. Protein was pooled, diluted to 25 mM NaCl and loaded onto a 1 ml SP HP™ column. Protein was eluted at 50 mM NaCl, tested for purity as described above, pooled and dialysed to buffer S for storage at -80°C. Protein concentrations were determined using bovine serum albumin standards and a BioRad protein assay.

Adenylyltransferase activity assays

For DRB0100, reaction mixture (20 µl) containing 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 5 mM $MgCl_2$, 1.25 µM α-[^{32}P]-ATP and the indicated amounts of protein were incubated for 15 minutes at 30°C. 20 µl of 2X Laemmli buffer were added, samples were heated for 5 minutes at 95°C and products were separated on a 12% standard SDS-PAGE. The intermediates were detected by autoradiography and the gel was stained by Coomassie Blue R250 to visualize the molecular weight markers. For LigA, the reaction mixture (10 µl) contained 50 mM Tris-HCl, pH 6.8, 5 mM dithiothreitol, 1 mM $MnCl_2$, 1 µg of protein and 0.1 µM [^{32}P]-NAD^+. The reaction was incubated for 15 minutes at 30°C, stopped with 10 µl of 2X Laemmli buffer, heated for 5 minutes at 95°C and loaded onto a 10% SDS-PAGE. Prestained markers were loaded to compare protein sizes. Free [^{32}P]-NAD^+ and ligase-AMP complexes were visualized by autoradiography.

Preparation of DNA substrates

The DNA substrate used to measure the ligation activity on a double-stranded substrate carrying a single-strand nick was prepared as described [33], the 19 nucleotide DNA strand was phosphorylated using γ-[^{32}P]-ATP and T4 polynucleotide kinase (New England Biolabs). Free γ-[^{32}P]-ATP was removed on a MicroSpin™ G-25 column (GE Healthcare). The sequences are presented in Table 3.

Ligation assays

The 5'-[^{32}P]-labelled DNA substrate was incubated for 30 minutes with the indicated amounts of recombinant protein at 30°C. Reactions were performed in a final volume of 10 µl containing 50 fmol of 5'-[^{32}P]-labelled DNA, 50 mM Tris-HCl, pH 6.8, 1 mM $MnCl_2$, 5 mM dithiothreitol and 1 mM ATP or 5 µM NAD^+ unless otherwise mentioned. The reactions were stopped by adding 10 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol), heated for 5 minutes at 95°C and products were separated on a 15% denaturing polyacrylamide gel containing 8 M urea and 15% formamide. After autoradiography, the signals were

quantified on a PhosphorImager using the ImageQuant software (Molecular Dynamics). Ligation was quantified by calculating the product/(product+substrate) ratio thus allowing a correction for loading errors. To determine the $K_{\text{Mnicked DNA}}$ value of the *D. radiodurans* NAD^+-dependent DNA ligase, the reactions contained 50 mM Tris-HCl, pH 6.8, 5 mM dithiothreitol, 1 mM $MnCl_2$, 5 μM NAD^+, 10-150 fmol of [^{32}P]-labelled nicked DNA substrate and 2 ng of enzyme. The reactions were incubated for 15 minutes at 30°C. The ligated products were quantified by PhosphorImager and $K_{\text{Mnicked DNA}}$ was calculated by Lineweaver-Burk plotting as a mean of 3 independent experiments.

Polynucleotide kinase assays

The indicated amounts of recombinant protein were incubated with 1 pmol of DNA substrate (kinase-DNA in Table 3) in a volume of 10 μl containing 50 mM Tris-HCl, pH 7.5, 0.25 mM $MnCl_2$, 5 mM dithiothreitol and 0.25 μCi of γ-[^{32}P]-ATP (GE Healthcare) for 30 minutes at 30°C. Reactions were stopped with 10 μl of loading buffer, heated for 5 minutes at 95°C and separated on a 15% denaturing polyacrylamide gel containing 8 M urea and 15% formamide. Signals of ^{32}P-DNA were visualized by autoradiography.

Authors' contributions

MB performed most of the experiments, did most of the cloning and protein purifications, analysed the data and wrote the manuscript. RB helped characterising the NAD^+-dependent DNA ligase. IS performed part of the cloning and protein purification. MB, IS and UH conceived of the study. UH helped analysing the data and preparing the manuscript. All authors read and approved the final version of the manuscript.

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Figure legends

Figure 1. Alignment and purification of two predicted *D. radiodurans* DNA ligases.

A. Alignment of eight colinear sequence elements in bacterial DNA ligases based on previous studies of DNA ligase motifs [7, 34] using CLUSTALW alignment [35]. The numbers of amino acids between the motifs are indicated. The alignment of motif VI is not shown for the ATP-dependent DNA ligases since the homology is very poor. Note that the putative ATP-dependent DNA ligase from *D. radiodurans* seems to lack also motif V. The conserved adenylated lysine residue is depicted in bold and labelled with an asterisk. Dr, *Deinococcus radiodurans*, Ec, *Escherichia coli*, Bs, *Bacillus subtilis*, Mt, *Mycobacterium tuberculosis*, Hi, *Haemophilus influenzae*. B. Predicted domain structures of *D. radiodurans* NAD^+-dependent DNA ligase (LigA) and ATP-dependent DNA ligase (DRB0100). The LigA protein scheme is based on homology searches using the NCBI conserved domain database and the SMART conserved domain database. OB, oligonucleotide-binding fold, Zn, zinc finger, HhH, helix-hairpin-helix motif 1, BRCT, BRCA1 C-terminal domain. C. LigA and DRB0100 and their corresponding adenylation mutants LigA K128A and DRB0100 K40A were purified over one metal affinity column and two ion exchange columns to near homogeneity as described in Methods. 3 µg of each protein were loaded onto a 10% SDS-PAGE and the gel was stained with Coomassie Blue R250.

Figure 2. DNA ligation and adenylyltransferase activities of the putative recombinant DNA ligases.

A. LigA wt and LigA K128A were incubated with [^{32}P]-NAD^+ and adenylyltransferase activity was detected by SDS-PAGE. Protein bands were visualized with Coomassie Blue R250 (left) and by autoradiography (right). B. DRB0100 wt and K40A were incubated with α-[^{32}P]-ATP. Protein-AMP complexes and free α-[^{32}P]-ATP were separated by SDS-PAGE. Proteins were stained with Coomassie Blue R250 (left) and detected by autoradiography (right). C. Titration of LigA wt and LigA K128A on a nicked DNA substrate. Indicated amounts of LigA wt and LigA K128A were incubated with the DNA substrate as described in the Methods section. [^{32}P]-labelled DNA oligonucleotides were visualized by autoradiography. D. Quantification of three independent experiments as shown in C. Error bars are given as the standard error of the mean.

Figure 3. Divalent cation requirements for LigA activity.

A. Titration of $MgCl_2$, $MnCl_2$ and $CaCl_2$. Ligation assays were performed with 60 fmol of LigA wt and increasing amounts of divalent cations, and quantified as described in Methods. Ligation activity obtained with 1 mM $MnCl_2$ was set as 100% and relative DNA ligation activity is shown as the average of 2 experiments.

B. Titration of $CoCl_2$, $NiCl_2$ and $ZnCl_2$. Ligation assays were performed as in A. Note that the ordinate has a scale of about 2 orders of magnitude lower than in A for better illustration of the divalent cation optima.

Figure 4. Purification of a putative PNKP from *D. radiodurans* and analysis of its 5' kinase and 3' phosphatase activities

A. Scheme of PNKP from *D. radiodurans* and *H. sapiens*. Protein domains are depicted according to predictions based on sequence similarities [36]. Schemes are not drawn to scale. HD, HD domain, kinase, polynucleotide kinase domain, HisB, histidinol phosphatase and related phosphatases domain. B. 3 µg of either *D. radiodurans* PNKP wt or PNKP R371K mutant were loaded onto a 10% SDS-PAGE and subsequently stained with Coomassie Blue R250. Both proteins were purified over 3 columns. Details are described in Methods.
C. Titration of the *D. radiodurans* PNKP wt and PNKP R371K mutant to compare their polynucleotide kinase activity on a 5'OH 25mer deoxyribose oligonucleotide. Different amounts of enzyme were incubated with the DNA substrate and γ-[^{32}P]-ATP as described in Methods. [^{32}P]-labelled 25mer was detected by autoradiography.

Tables

Table 1. PCR primer sequences used in this study

Primer name	Used for	Sequence (5'-3')
DRB0100F	cloning of DRB0100wt into pRSETb	CGC*GGATCC*GATGCGAGTCAAATACCCTTC
DRB0100R	cloning of DRB0100wt into pRSETb	CGC*GGATCC*GTCATGACTGCTCCTGGCG
DRB0100_mutF	introduction of K40A mutation into DRB0100	CGTCGTGACCGAG**GC**GCTCGACGGCG
DRB0100_mutR	introduction of K40A mutation into DRB0100	CGCCGTCGAGC**GC**CTCGGTCACGACG
DR2069F	cloning of DR2069 wt into pRSETb	CGC*GGATCC*GATGCGTTACCCTGGGCGC
DR2069R	cloning of DR2069 wt into pRSETb	CGC*GGATCC*GTCAGCTTTCAGCGGGGGC
mut_DR2069F	introduction of K128A mutation into DR2069	CCGGCGAGCTG**GC**AATCGACGGCCT
mut_DR2069R	introduction of K128A mutation into DR2069	CAGGCCGTCGATT**GC**CAGCTCGCCGG
DRB0098F	cloning of DRB0098 into pRSETb	CGC*GGATCC*GATGAACCGCAAAAACCGTAC
DRB0098R	cloning of DRB0098 into pRSETb	CGC*GGATCC*GTCAGGAGGTAGATGAGGGCAG
98_R371LF	introduction of R371K mutation into DRB0098	GGTCAGCTCGGAGCA**AAA**ATCAGCGGGAGAG AGC
98_R371LR	introduction of R371K mutation into DRB0098	GCTCTCTCCCGCTGAT**TTT**TGCTCCGAGCTGA CC

All oligonucleotides were desalted and used in a final concentration of 0.4 µM. T7 sequencing primers can be found at the Microsynth webpage. Bold bases represent those exchanged in the site-directed mutagenesis. Restriction sites are shown in italics.

Table 2. K_M values of prokaryotic NAD^+-dependent DNA ligases

Organism	T [°C]	K_M [nM]	Reference
D. radiodurans	30	105±16	This work
E. coli	18	179	Georlette *et al.*, 2000
E. coli	30	702	Georlette *et al.*, 2000
E. coli	45	2040	Georlette *et al.*, 2000
P. haloplanktis	4	165	Georlette *et al.*, 2000
P. haloplanktis	18	296	Georlette *et al.*, 2000
P. haloplanktis	25	631	Georlette *et al.*, 2000
T. scotoductus	45	236	Georlette *et al.*, 2000
T. scotoductus	60	465	Georlette *et al.*, 2000

K_M values for nicked DNA substrates. Details for K_M determination of *D. radiodurans* NAD^+-dependent DNA ligase are described in Methods. K_M is the mean of 3 independent experiments and the error is given as standard error of the mean.

Table 3. Oligonucleotide sequences used to prepare DNA substrates for the enzyme assays performed in this study

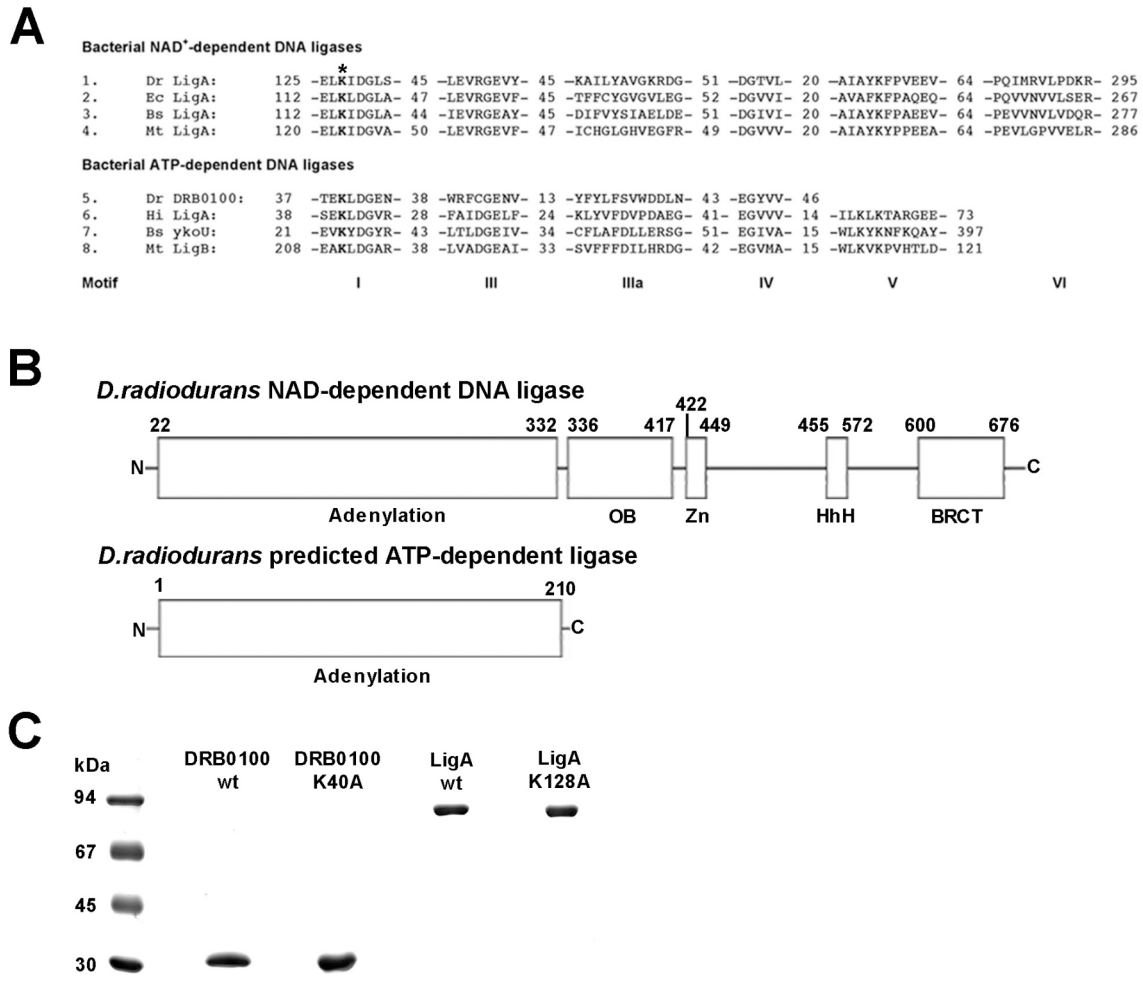
Oligonucleotide	Length (nt)	Sequence (5'-3')
RNA-5' nick	19	CAGCAGCAAAUGAAAAAUC
DNA-5' nick	19	CAGCAGCAAATGAAAAATC
RNA-3' nick	25	CCUGCAACAGUGCCACGCUGAGAGC
DNA-3' nick	25	CCTGCAACAGTGCCACGCTGAGAGC
DNA- 3'P nick	25	CCTGCAACAGTGCCACGCTGAGAGC-P
DNA-opposite	46	AGATTTTTCATTTGCTGCTGGCTCTCAGCGTGGCACTGTTGCAGGC
Kinase-DNA	25	GCTTTCCGAGTACCGGGGTCTTCCG

All oligonucleotides were PAGE purified and labelled as described in the Methods section. P stands for a 3' phosphate group.

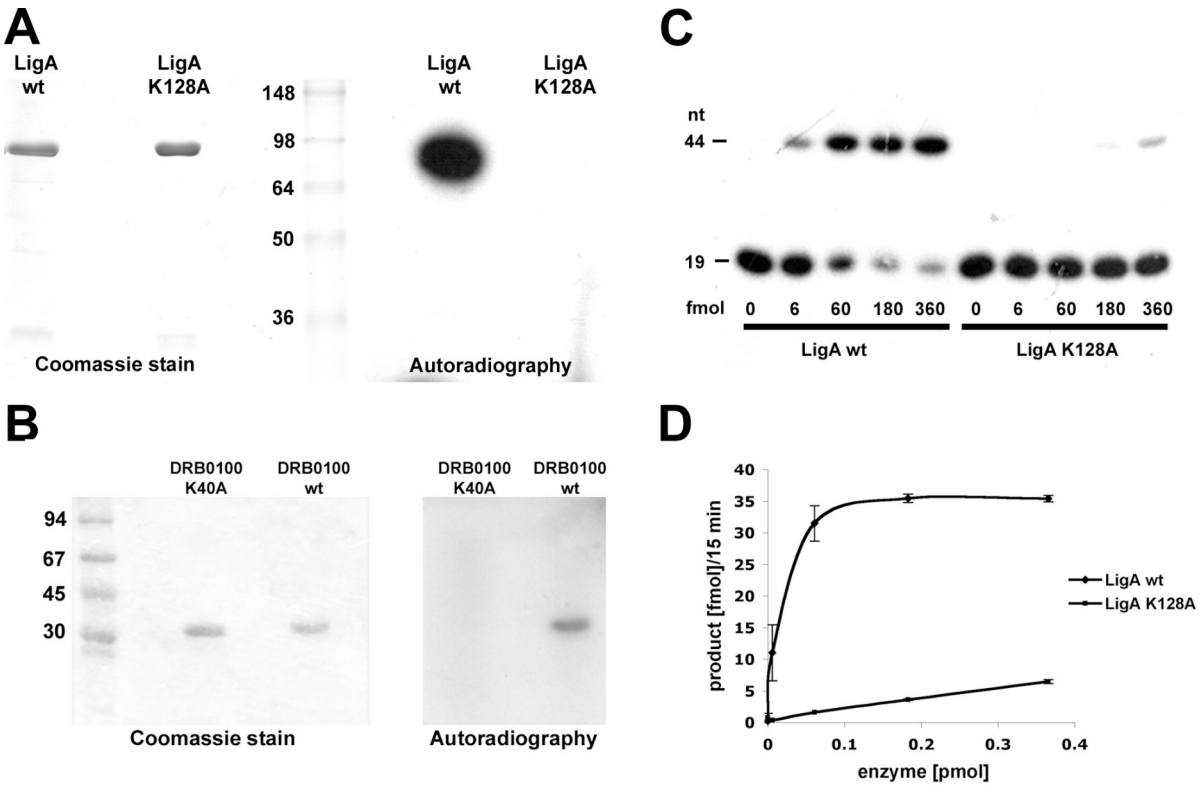
Additional files:

Additional file 1
File format: PDF
Title: Factors tested to detect DNA ligation activity of the DRB0100 gene product
Description: This table lists the various buffer conditions and proteins tested in DNA ligation assays for the DRB0100 gene product.

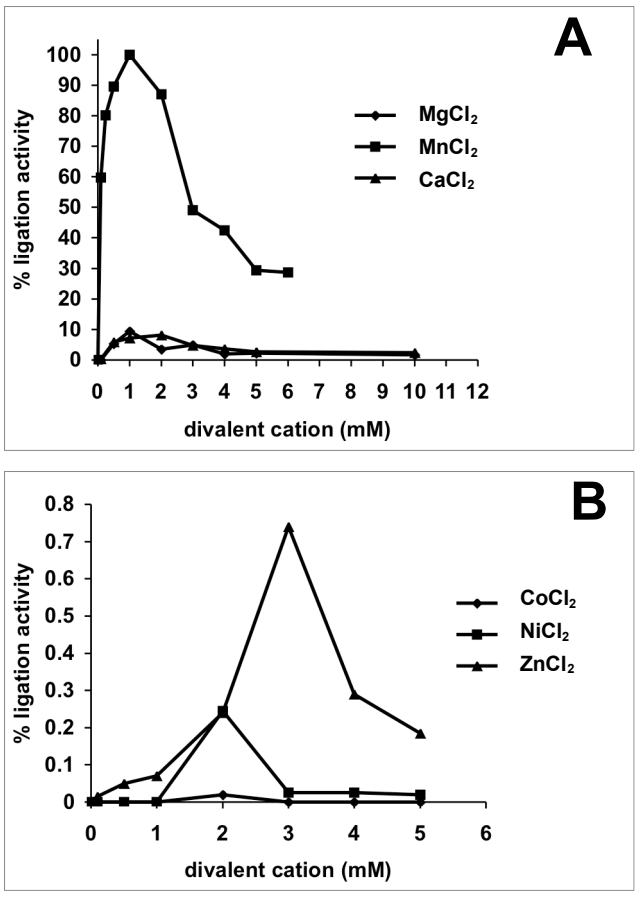
Additional file 2
File format: PDF
Title: Ligation substrates tested to detect DNA ligation activity of the DRB0100 gene product
Description: This table shows the sequences of the different oligonucleotides that were used to prepare ligation substrates for the DRB0100 gene product.



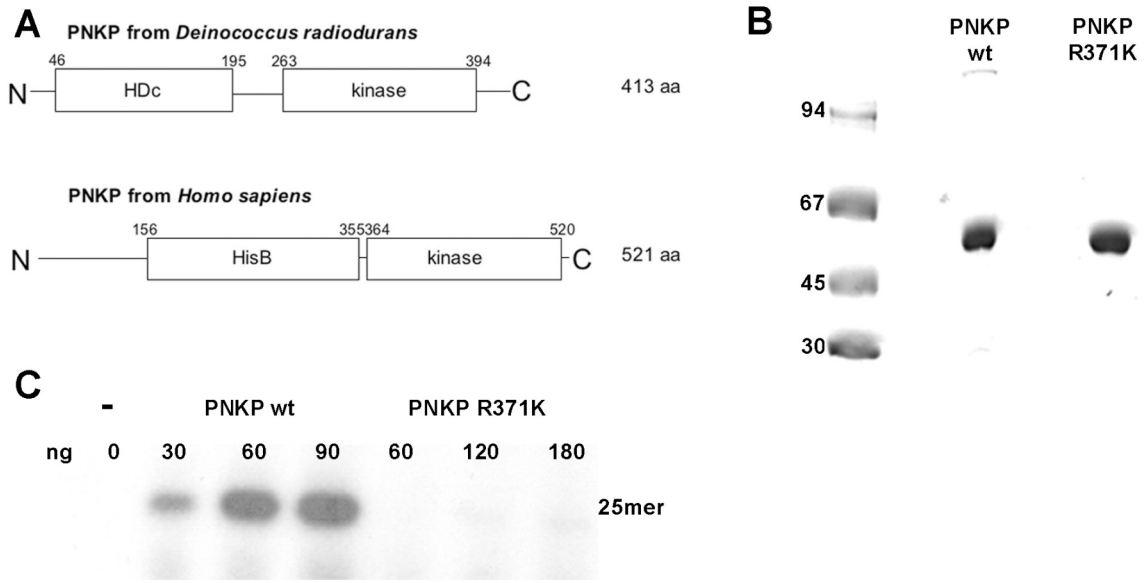
Blasius *et al.*, Figure 1



Blasius *et al.*, Figure 2



Blasius *et al.*, Figure 3



Blasius *et al.*, Figure 4

7 FURTHER UNPUBLISHED DATA

7.1. Selected unpublished results

1.1.1 Deinococcus radiodurans LigA

An antibody against the *D. radiodurans* LigA protein was produced in rabbit (see Methods in 7.2.) and tested for sensitivity as well as for specificity. The polyclonal antibody could specifically recognise the recombinant LigA protein in total *E. coli* extracts (data not shown) and could be used in a standard Western blotting experiments to detect several ng of purified protein (Figure 3A). Next, it was tested whether the polyclonal antibody would recognise the native LigA protein in total *D. radiodurans* extracts. A clear signal was detected in 2.5 µg of total cell extract, which migrated at the expected position (slightly faster than the recombinant protein due to the absence of the tag sequence) (Figure 3B). However, further experiments are required to confirm this finding. Polyclonal antibody was also produced against the *D. radiodurans* protein PprA. The antibody was able to detect the recombinant protein (data not shown) as well as a band of the expected size in *D. radiodurans* extracts (Figure 3B). The antibody has been produced like the LigA antibody, but has so far not been used for further experiments.

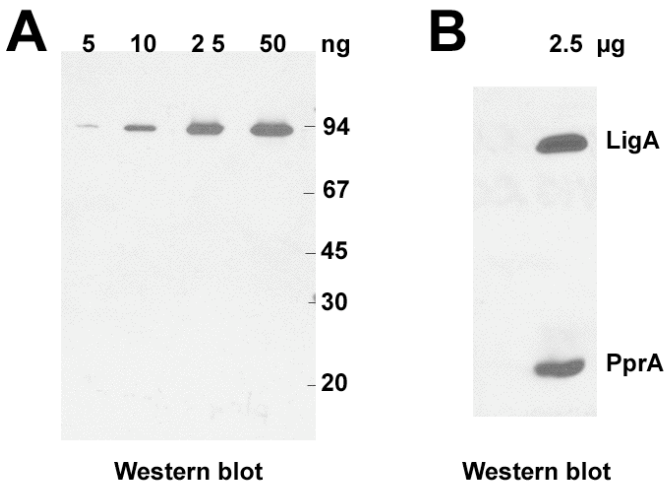


Figure 3. A specific antibody recognising *D. radiodurans* LigA. A. Either 5, 10, 25 or 50 ng of recombinant LigA wt protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with rabbit serum against the *D. radiodurans* LigA protein and was further treated as described in Methods. **B.** 2.5 µg of total *D. radiodurans* cell extract were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was cut horizontally and the upper half was incubated with rabbit serum against *D. radiodurans* LigA, whereas the lower part was incubated with rabbit serum against *D. radiodurans* PprA.

Total *D. radiodurans* extracts from non-irradiated cells were used for DNA ligation experiments. The aim was to test for ATP-dependent DNA ligation. For this reason, total *D. radiodurans* extract was incubated with a nicked DNA substrate either in presence of absence of NAD^+ or ATP as an AMP-donor. Due to a fraction of pre-adenylated DNA ligase in the extract, some DNA ligation was observed even in the absence of an additional AMP-donor (Figure 4, lane 3). This basal activity could be strongly increased by addition of NAD^+ (lanes 2, 5 and 6), indicating the presence of an NAD^+-dependent DNA ligase in the extract. Additon of 1 mM ATP however, did not increase the formation of DNA ligation product (Figure 4, lane 4), suggesting that either no ATP-dependent DNA ligase is present in unstressed *D. radiodurans* cells, that it is only present in very small amounts or that it is unstable or inactive under conditions used in this experiment. A similar experiment was performed using total extracts from irradiated *D. radiodurans* cells with the same outcome.

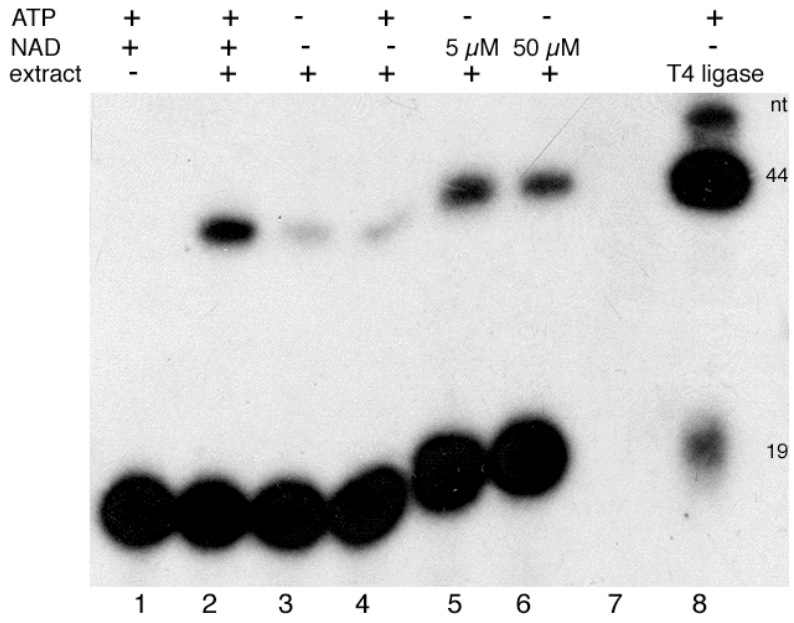


Figure 4. DNA ligation using total *D. radiodurans* extracts. A nicked DNA substrate was incubated with extract buffer (lane 1), or 244 ng total *D. radiodurans* extract (lanes 2-6) either with 1 mM ATP (lane 4), 5μM NAD^+ (lane 5), 50 μM NAD^+ (lane 6), a combination of 5 μM NAD^+ and 1 mM ATP (lane 2) or without an AMP-donor (lane 3). As a positive control for DNA ligation, T4 DNA ligase was incubated with the nicked substrate in presence of 1 mM ATP (lane 8). Non-ligated DNA substrate (detected as 5'-labelled 19mer) and DNA ligation product (internally labelled 44mer) were separated in a 15% denaturing polyacrylamide gel and visualised by autoradiography.

In order to estimate the DNA binding properties of the DNA ligase electromobility shift assays (EMSA) were performed by using the duplex DNA substrate with a single nick (for sequences, see 6.2). The nicked DNA substrate was first used with the label at the 5' end of the 25mer and not at the nick to prevent ligation, but the ligase did not bind to this substrate without a 5' phosphate at the nick (data not shown). Therefore the nicked substrate with the radioactive phosphate at the nick was used and NAD^+ was omitted from the reactions. The K128A mutant of LigA, that was unable to carry out self-adenylation, nicely shifted the DNA, whereas the shift was much weaker for the same amount of wild-type LigA (Figure 5A). This difference has been observed before and could be explained by pre-adenylation of the over-expressed enzyme in *E. coli* [50]. Pre-adenylation would allow a single-turnover of each enzyme molecule leading to only a very transient complex in the case of the wild-type enzyme, whereas the non-adenylated mutant could form a stable DNA-enzyme complex. To confirm this hypothesis the same EMSA reactions were performed and loaded on a 15% denaturing gel to detect possible ligation products. As expected for the wild-type enzyme the DNA substrate was almost completely ligated without the addition of NAD^+ to the buffer, whereas the K128A did not ligate the DNA substrate under these conditions (Figure 5B).

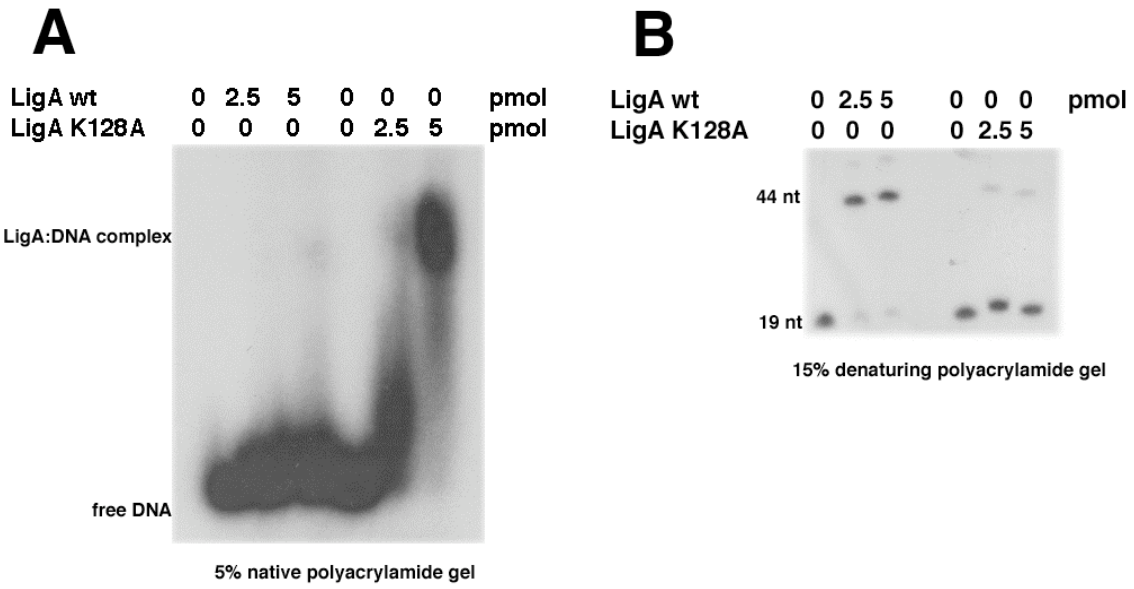


Figure 5. The LigA K128A mutant forms a more stable complex with nicked DNA than the LigA wildtype. (**A**). The LigA K128A mutant can bind to the nicked DNA in the absence of NAD^+ (lanes 5 and 6), whereas the wild-type DNA ligase does not form a stable complex under these conditions (lanes 2 and 3). In lanes 1 and 4 enzyme storage buffer was added instead of enzyme. (**B**). Reactions were performed as in A but were loaded on a 15% denaturing gel to detect the DNA ligation product as an internally ^{32}P-labelled 44mer.

To see whether adenylation of LigA could also be detected in total extracts from *D. radiodurans*, we incubated either 10 or 20 µg of extract with ^{32}P-NAD$^+$. The samples were then separated on an SDS-polyacrylamide gel and putative ^{32}P-AMP-protein complexes were visualised by autoradiography. As expected a single protein band was identified, which corresponded approximately to the position of LigA. Although further experiments are required to confirm, that the signal indeed comes from an adenylated LigA protein, this possibility seems the most likely. An adenylyltransferase assay was also performed by using *D. radiodurans* cell extracts and γ-[^{32}P]-ATP. At least three distinct bands were detected on the autoradiography (data not shown) and no final conclusion could be made as these bands could as well result from protein phosphorylation or unspecific binding to ATP.

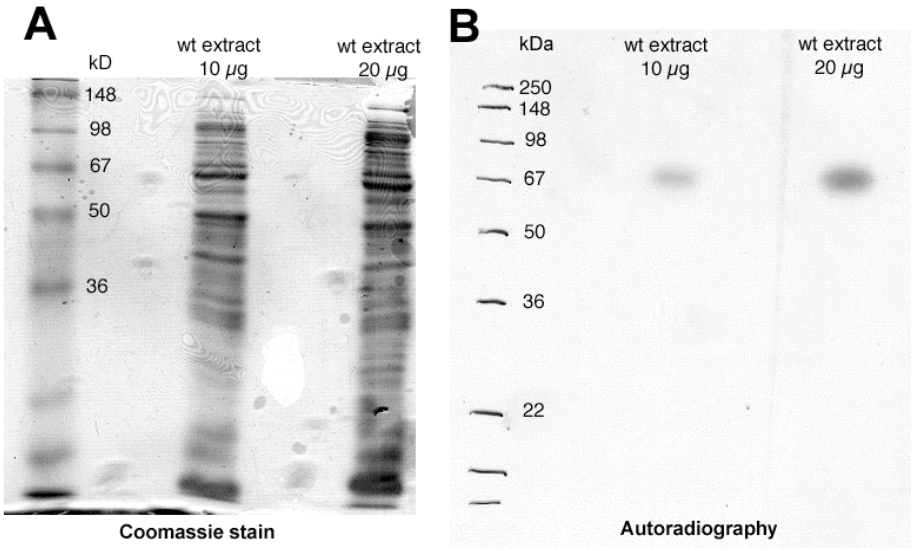


Figure 6. Adenylyltransferase assay with total *D. radiodurans* extracts. A. 10 or 20 µg of total *D. radiodurans* extract were incubated with ^{32}P-NAD$^+$, separated by SDS-PAGE and subsequently stained by Coomassie R250. **B**. Autoradiography of the gel shown in A.

7.1.2. Deinococcus radiodurans polynucleotide kinase substrate specificity

It was tested whether the polynucleotide kinase of *D. radiodurans* has s specificity for blunt or recessed DNA ends. T4 PNK, for example, prefers RNAs or DNAs with 5' overhangs and is poorly phosphorylating blunt duplex DNA termini or 5'OH ends at the junction of a 3' ss overhang [51] and the mammalian polynucleotide kinase prefers to phosphorylate recessed 5' ends in a duplex DNA [52]. The ability of *D. radiodurans* PNKP to phosphorylate blunt ends, ss 5' overhangs or single-stranded 3' overhangs was tested. Plasmid substrates were prepared by restriction of pRSETb DNA with a restriction endonuclease that cleaves the plasmid once, leaving either a 4-nucleotide 5' overhang (BamHI), a 4-nucleotide 3' overhang (SacI), or a blunt duplex (PvuII), followed by treatment with calf intestine phosphatase, which removes all 5'-phosphate groups. The DNA substrates were purified from an agarose gel and incubated with *D. radiodurans* PNKP in the presence of γ-[^{32}P]-ATP. The DNAs were analysed by agarose gel electrophoresis and visualised by ethidium bromide staining (Figure 7, top panel). Transfer of [^{32}P]-phosphate to the 5'OH ends of the DNA was visualised by autoradiography (Figure 7, bottom panel). *D. radiodurans* PNKP could phosphorylate all DNA substrates with similar efficiencies. The substrate with a 5' overhang seems to be less favoured compared to the others, but a more quantitative experiment would be required to conclude in more detail about the substrate preferences of the enzyme. In summary, *D. radiodurans* PNKP was able to phosphorylate different DNA substrates *in vitro*.

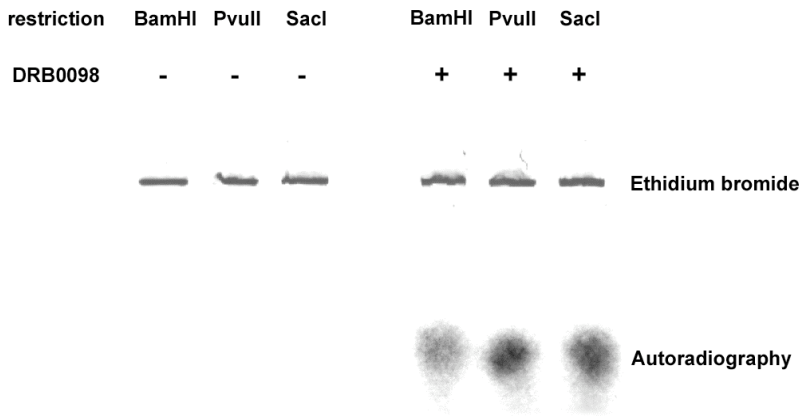


Figure 7. End specificity of the polynucleotide kinase activity of the *D. radiodurans* PNKP. *D. radiodurans* PNKP or enzyme storage buffer were incubated with γ-[^{32}P]-ATP and 5'-OH terminated pRSETb DNA generated by restriction with BamHI, SacI, or PvuII. The DNA was then analysed by agarosel gel electrophoresis and Ethidium bromide staining (top panel), and radiolabelled DNA was visualised by autoradiography (bottom panel).

Next, the activity of the polynucleotide kinase on ss or ds DNA ends in presence of either Mn(II) or Mg(II) was compared. As expected, the *D. radiodurans* polynucleotide kinase prefered Mn(II) as a metal cofactor and ss DNA is a better substrate than ds DNA (Figure 8).

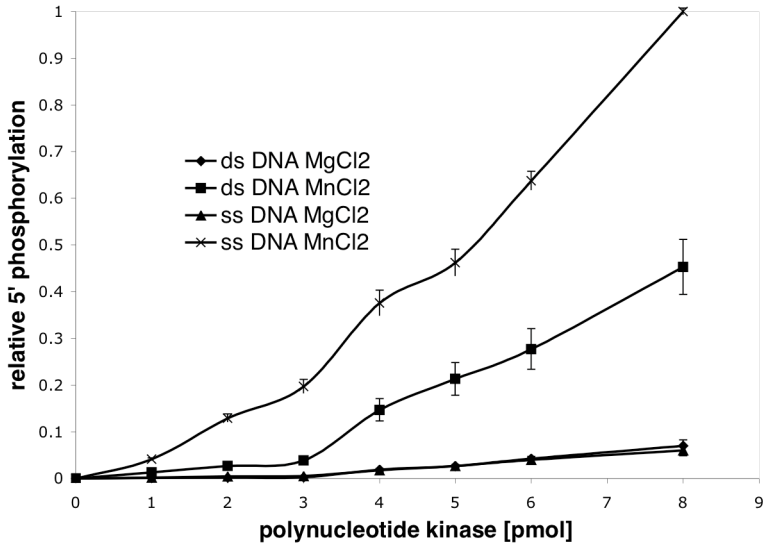


Figure 8. *D. radiodurans* polynucleotide kinase prefers Mn(II) as a metal cofactor and ss DNA is a better substrate than ds DNA. Relative polynucleotide kinase activity of DRB0098 on either single- or double-stranded DNA ends was measured in the presence of either 5 mM $MgCl_2$ or 0.5 mM $MnCl_2$. Error bars indicate the standard error of the mean from 3 independent experiments.

Various DNA binding experiments were also performed to test whether the *D. radiodurans* polynucleotide kinase would preferentially bind to DNA with ss overhangs or DNA possessing 5' OH termini, but two different band shifts were seen with most substrates no conclusion could be drawn from these experiments. It is well possible that the enzymes bind to DNA termini in general, but further experiments are definitely required to draw safe conclusions.

7.1.3. The Deinococcus radiodurans DRB0099 gene product binds ADP-ribose

The third protein of the same putative DNA repair operon (further described in 6.2), DRB0099, has no assigned function and shows only weak similarities to the histone variant MacroH2A. Histone MacroH2A is an unusual core histone, consisting of a very conserved H2A region and a large non-histone region. Sequence comparison of the non-histone regions found in bacteria and RNA viruses suggest that this domain originated prior to the appearance of eukaryotes [53]. As the domain is found in many otherwise unrelated proteins and is present in bacteria, archaea, viruses and eukaryotes it probably has a ubiquitous and important cellular function. The exact function of this domain is not known, but it seems to be a high-affinity ADP-ribose binding module [54]. ADP-ribosylation is an important post-translational protein modification playing a role in many cellular processes such as DNA repair and transcription. Mono-ADP-ribosylation of histones is a known response to DNA damage and plays a role in the recruitment of DNA repair complexes. For human cells, clear evidence exists that such histone modifications play a crucial role in DNA repair processes (reviewed in [55]). The MacroH2A domain is the first protein module that specifically binds the ADP-ribose moiety. The presence of a MacroH2A domain in the DRB0099 protein therefore suggested that the protein binds to ADP-ribose. Preliminary experiments indeed show a significant binding to ADP-ribose monomers (Figure 9) that is in the range of other ADP-ribose binding proteins [54]. However, further work is required to confirm the specificity of this interaction and to define a functional role in DNA repair. It could be tested whether DNA or proteins in *D. radiodurans* can be ADP-ribosylated in general and if yes, whether this is a mechanism to regulate interactions.

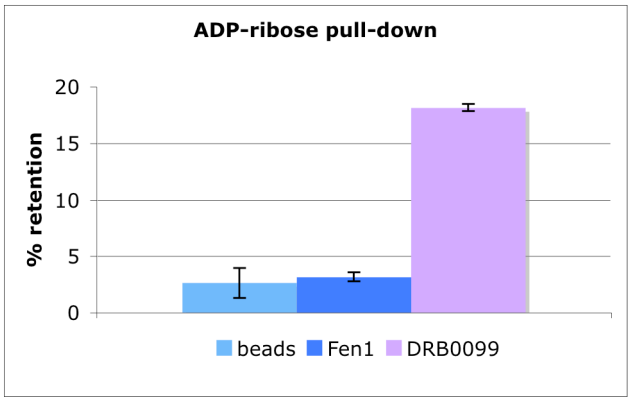


Figure 9. The operon protein DRB0099 binds ADP-ribose. 15 nMol of either His-tagged Fen1 or His-tagged DRB0099 were bound to Ni-NTA-agarose and incubated with 15 nMol of ADP-ribose. Unbound ADP-ribose was measured in a spectrophotometer at 260 nm and ADP-retention was calculated as the difference in absorption between the unbound supernatant and 15 nMol ADP-ribose. Pull-downs were performed in triplicate and error-bars indicate the standard error of the mean.

7.2. Material and Methods

7.2.1 Antibody production and Western blotting

Rabbit polyclonal antibodies against *D. radiodurans* LigA and *D. radiodurans* PprA were raised in rabbits by injecting 3 times approximately 200 µg of purified recombinant protein.

For Western blotting, proteins were separated on a 10% SDS-gel, transferred to nitrocellulose for 1.5 hours with constant application of 100 V. The membrane was incubated with TBST/5% (w/v) milk powder for 45 minutes, then overnight with a 1:5000 dilution of the corresponding rabbit polyclonal antibody. After 3 washes with TBST the membrane was further incubated with ECL™ Anti-rabbit IgG (from donkey, GE Healthcare) in a 1:5000 dilution for 1 hour. After 3 more washes, the membrane was developed using the Uptilight HRP blot reagent (optima) and exposed to an X-ray film (Contatyp) for several seconds.

7.2.2 Preparation of D. radiodurans total extracts

D. radiodurans cells were grown at 30°C in 2X TGY medium (1% tryptone, 0.2% glucose, and 0.6% yeast extract). Cell pellets were dissolved in lysis buffer (50 mM Tris-HCl, 500 mM KCl, 2 mM EDTA, 2 mM DTT, bestatin, pepstatin and leupeptin (Bachem, 1µg/ml final concentration). 3 mg of glass beads were added and cells were lysed by vortexing 8 times 20 seconds, leaving the lysate 30 seconds on ice between the vortexing steps. The lysate was centrifuged twice at 700g and the supernatant was dialysed overnight to extract buffer (20 mM Tris-HCl, pH 7.5, 20 (v/v) % glycerol, 1 mM DTT, 0.5 mM EDTA, 0.2 mM PMSF and 25 mM NaCl). Protein concentrations were determined using a standard Bradford assay and the extracts were stored at -80°C until further use.

7.2.3 DNA ligation assay

The 5'-^{32}P-labelled nicked DNA substrate (for sequences see 6.2) was incubated for 15 minutes with 244 ng of total extract or extract buffer at 30°C. Reactions were performed in a final volume of 10 µl containing 50 fmol of 5'-^{32}P-labelled DNA, 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT and 1 mM ATP or 5-50 µM NAD^+ unless otherwise mentioned. The reactions were stopped by adding 10 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and the products were separated on a 15% denaturing polyacrylamide gel containing 8 M urea and 15% formamide. The 19mer substrate and the 44mer product were visualised by autoradiography.

7.2.4 DNA binding assay

As a substrate for the DNA binding experiments a double-stranded oligonucleotide containing a single nick was used. This substrate was also used for the DNA ligase activity assays. Binding reactions of 20 µl contained 50 mM Tris-HCl, pH 6.8, 1 mM $MnCl_2$, 5 mM DTT, 50 fmols ^{32}P-labelled substrate and the indicated amounts of purified proteins. Reactions were incubated for 30 minutes at room temperature. The reactions were mixed with 20 µl of loading buffer (50 % sucrose, 0.01% bromphenol blue, 0.01% xylene cyanol), loaded on a native 5% polyacrylamide gel in 0.5X TBE and run for 3 hours at 5 mA. The band shifts were detected by autoradiography.

7.2.5 Polynucleotide kinase activity assay

For testing the end specificity of the *D. radiodurans* PNKP, pRSETb plasmid was linearised with BamHI, SacI, or PvuII, leading to 4-nucleotide 3' overhangs, 4'-nucleotide 5' overhangs or blunt ends, respectively. Restriction enzymes were heat inactivated, the DNA was treated with calf intestine phosphatase and purified from an agarose gel using a gel purification kit (Qiagen). A polynucleotide kinase activity reaction of 10 µl contained 800 ng of *D. radiodurans* PNKP, 50 mM Tris-HCl, pH 7.0, 5 mM DTT, 0.25 mM $MnCl_2$, 0.3 µCi of γ-[^{32}P]-ATP and 100 ng of linear DNA. Incubation was done for 30 minutes at 30°C, followed by an agarose gel electrophoresis. Total DNA was visualised with Ethidium bromide and radiolabelled DNA was detected by autoradiography. For comparing the phosphorylation of ss to ds DNA ends, we incubated increasing amounts of *D. radiodurans* polynucleotide kinase with 1 pmol of either ss or ds oligonucleotides of 25 nucleotides length possessing 5'-OH ends. Reactions were performed in a final volume of 10 µl containing 1 pmol of the corresponding DNA, 50 mM Tris-HCl, pH 7.5, 0.25 mM $MnCl_2$, 5 mM DTT and 0.25 µCi of γ-[^{32}P]-ATP (GE Healthcare) for 30 minutes at 30°C. reactions were stopped by adding 10 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and the radiolabelled 25mer was analysed on a 15% denaturing polyacrylamide gel containing 8 M urea and 15% formamide. The product was quantified using a PhosphoImager and the ImageQuant Software.

7.2.6 ADP ribose pull-down

15 nmol of a His-tagged protein were bound to Nickel beads (Pro-Bond, Invitrogen) pre-equilibrated in phosphate buffer saline for 30 minutes at 4°C. The supernatant was removed and 1 ml of a 15 µM ADP-ribose solution (Sigma-Aldrich) in phosphate buffered saline was added for 30 minutes at room temperature. The supernatant containing the unbound ADP-ribose was measured in a spectrophotometer at 260 nm. % ADP-retention was calculated as difference between input and ADP-ribose in the supernatant.

7.3. Discussion of unpublished results

By different means it was shown that *D. radiodurans* possesses a NAD^+-dependent DNA ligase, which efficiently joins DNA nicks (see 6.2 and 7.1.1). Although a second ATP-dependent DNA ligase has been predicted from genome sequence comparisons, no indications for the presence of such an ATP-dependent DNA ligase in *D. radiodurans* cells could be found (7.1.1., Figure 4).

Although the *in vivo* role of the putative DNA repair operon DRB0098-DRB0100, coding for the putative ATP-dependent DNA ligase and two additional proteins, remains unclear, some interesting data has been obtained for the two products of the genes DRB0098 and DRB0099. DRB0098 encodes a polynucleotide kinase (further details are given in 6.2). This polynucleotide kinase can phosphorylate different DNA ends, such as blunt ends or overhangs (Figure 7). However, the enzyme prefers ss DNA termini over ds DNA ends and is much more active with Mn(II) as a cation cofactor than with Mg(II). A preference for ss DNA ends is consistent with the current models of DNA strand-break repair and the preference for Mn(II) is similarly unsurprising, considering the very high intracellular Mn(II) concentration in *D. radiodurans*.

8 CONCLUSIONS AND PERSPECTIVES

This thesis work describes novel DNA repair enzymes from *D. radiodurans* with a possible involvement in DNA strand-break repair. A structure-modulated nuclease containing a Pol X domain has been characterised and a role for this enzyme in DNA strand-break repair has been shown *in vivo*. This protein is the first prokaryotic member of the Pol X family that has been characterised and the first member of this Pol family that is associated with a nuclease activity. As expected, the nuclease activity is strongly stimulated by low concentrations of Mn(II) and mutation of two conserved glycine residues in the Pol X domain impairs the structure-modulated nuclease activity. Deletion of the corresponding gene in D. *radiodurans* leads to a delay in DNA double-strand break repair. The sensitive phenotype can be restored by expressing the wild-type protein *in trans*, but not by expressing the glycine mutant, confirming an important role of the Pol X domain *in vivo*. It would be interesting to analyse the structure of this bacterial protein and compare it to eukaryotic family X Pols. Furthermore, the protein could well be part of a DNA repair complex and protein-interaction as well as genetic studies could identify possible partners acting in the same pathway.

In addition to the structure-related nuclease, a DNA ligase and a polynucleotide kinase have been shown to perform DNA ligation of 3'-phosphorylated DNA nicks *in vitro*. *D. radiodurans* encodes a standard bacterial NAD^+-dependent DNA ligase. The protein has been expressed in *E. coli*, purified and characterised biochemically. The DNA ligase is specifically adenylated at lysine 128 and can efficiently ligate DNA nicks in the presence of Mn(II). A second ATP-dependent DNA ligase has been predicted based on weak similiarities with human DNA ligase III [26]. Although the recombinant protein is specifically adenylated at lysine 40 using ATP as an AMP-donor, the enzyme does not perform any DNA or RNA ligation in *vitro*. It is possible that the enzyme lacks another subunit or that the correct conditions for enzymatic activity have been missed. Protein interaction studies could eventually confirm the hypothesis of the missing subunit. In addition, the protein is surprisingly small and cannot bind to DNA. Since the genome sequence of *D. radiodurans* has previously been shown to contain errors [56], the region encoding the putative ATP-dependent DNA ligase (DRB0100) has been resequenced. The published sequence could be confirmed, thus excluding the possibility of a truncated protein (M. Blasius and U. Hübscher, unpublished data). The DRB0100 gene product is currently also tested in another context: the adenylation domain of *D. radiodurans* LigA has been replaced by DRB0100 and the chimeric protein will be purified and tested for a potential DNA ligation activity.

The gene DRB0098 encoded by *D. radiodurans* has been identified as a polynucleotide kinase. To our knowledge, this is the second polynucleotide kinase identified from a

prokaryote. The enzyme has been characterised biochemically, revealing a preference for ss DNA ends and Mn(II) as a divalent cofactor. Further *in vivo* work is required to fully understand the role of this enzyme in radioresistance. To analyse this, the gene should be deleted from the *D. radiodurans* genome and the cells should be screened for a possible phenotype, such as a DNA repair defect. It also remains to be elucidated whether the enzyme in addition to its polynucleotide kinase activity possesses a 3' phosphatase activity. An enzymatic mutant should be created as it has been done for the kinase activity (see 6.2).

Our preliminary data further suggest the presence of an ADP-ribose binding protein in *D. radiodurans*. This protein is part of the putative DNA repair operon DRB0098-DRB0100. Future studies should address the question whether ADP-ribosylation occurs in *D. radiodurans* and whether it plays an important function for example in regulating and coordinating the various DNA repair systems. A first approach could investigate whether ADP-ribosylated proteins are present in *D. radiodurans* cell. Total extracts could be incubated with ^{32}P-NAD and analysed via gelelectrophoresis and autoradiography. It is also possible to incubate the cell extract with etheno-NAD$^+$. ADP-ribosylated proteins can then be detected via a specific antibody [57] or isolated using an affinity column with the same antibody. Mass spectrometry analysis might allow further conclusions on whether proteins are covalently modified. Once candidate proteins have been identified, their modification could be confirmed by direct ADP-ribosylation assays (for example as in [58]). Another interesting aspect is that ADP-ribosylation can occur either as mono-ADP-ribosylation or as poly-ADP-ribosylation. The latter has never been shown to exist in prokaryotes. No homolog of poly-ADP-ribose polymerase has been identified in the *D. radiodurans* genome, and western blotting of total extracts and incubation with polyclonal antibody against human poly-ADP-ribose polymerase 1, showed a distinct band of approximately 80 kDa that might well be LigA due to some sequence similarity. Very similar results have been published for several other bacteria although there the main detected signal had a size of approximately 50-60 kDa [59]. In order to look for ADP-ribose polymers in *D. radiodurans*, an immunoblot could be done using an antibody against poly-ADP-ribose.

All identified enzymatic activities are strongly stimulated by Mn(II) confirming a crucial role for Mn(II) in DNA repair. Many predicted enzymes with unique architectures and novel features still need to be characterised in order to fully understand DNA repair in *D. radiodurans*. Also several other aspects require further work and might impact the picture of bacterial DNA repair and DNA damage signalling in general:

For instance, irradiated *D. radiodurans* cultures show a dose dependent growth lag. This inhibition of replication is similar to eukaryotic DNA damage checkpoints that allow time for

DNA repair and “cell cleaning”. Some indications for bacterial checkpoints have been observed, like cell-division inhibitors in *E. coli* [60] or a checkpoint for sporulation in *B. subtilis* [61], and are frequently discussed. Nevertheless, a bacterial checkpoint has not yet been formally established.

Several protein families have been implicated in removing damaged protein and DNA fragments and precursors, e.g. the NUDIX family of hydrolases, the members of which prevent incorporation of mutagenic DNA precursors [1]. This protein superfamily is strongly expanded in *D. radiodurans*, encoding 23 proteins that contain 25 individual MutT domains, and its members are probably able to hydrolyse a variety of nucleoside diphosphate derivates [6, 17, 62-72]. However, not all family members have been characterised to date.

Several hints exist that indicate an important role of RNA repair and even a link between DNA repair and RNA synthesis has been suggested [27]. The possibility exists that RNA intermediates are generated during DNA repair. RNA incorporation may be beneficial when levels of dNTPs are low as for example during stationary phase. Short stretches of RNA maybe replaced and corrected at a later stage. Although a direct role for RNA in double-strand break repair has not yet been proven, recent data suggest that yeast cells can use RNA as a repair template *in vivo*, probably via DNA synthesis across a RNA template by DNA polymerase δ [73]. A similar possibility exists in prokaryotes, where pol I has been shown to possess RNA-templated DNA synthesis *in vitro* [74].

Interesting insight in evolution towards radioresistance was gained from comparisons with the genome of *Thermus thermophilus*, a close relative from the same eubacterial phylum [9], including many indications that horizontal gene transfer played a major role for the evolution of bacteria and archaea [6, 75].

Further analysis of different genome, proteome and transcriptome comparisons of radioresistant species could allow for a definition of a minimal set of genes that are required for substantial radioresistance. And with respect to DNA repair, it would be interesting to reconstitute the repair of different kinds of DNA strand breaks using purified proteins *in vitro*. Similar approaches have been used for human DNA repair enzymes (reviewed in [76] and [77]) and Mycobacterium tuberculosis [20] allowing further conclusion about a basic set of DNA repair component for a specific DNA damage. Along this line, it should be also possible to design a set-up to differentiate between non-homologous end-joining and homologous recombination. Especially, since it is still not clear whether a non-homologous end-joining pathway exists in *D. radiodurans*. Tagging one of the candidate players, expressing it in *D. radiodurans* and then isolating it by tandem affinity purification could identify DNA repair complexes. Similarly, larger amounts of *D. radiodurans* cells could be irradiated, the

damaged DNA could be isolated and used to fish specific DNA repair enzymes and DNA damage recognising proteins from irradiated extracts. Irradiated cell extracts could also be fractionated and the fractions could be tested for their DNA repair capacity *in vivo*, either alone or with the addition of recombinant proteins.

As it has been done for *E. coli* [78], *D. radiodurans* strains could be created that either constitutively or upon induction express an restriction enzyme that cuts at multiple sites in the *D. radiodurans* genome. This would allow the induction of enzymes involved in DNA strand-break repair without high doses or γ-irradiation and strongly facilitate the isolation of novel DNA repair complexes, because large amounts of cells could be grown. However, this approach would only lead to “clean” DNA breaks, not perfectly mimicking the broad variety of DNA lesions caused by high doses of γ-radiation. Another problem would be that all copies of the chromosomes would theoretically be broken at the same site, making homologous recombination very difficult. This problem has been solved in *E. coli* where a DNA double-strand break is targeting only one copy of two essential identical sequences [79]. A similar system might be applied to *D. radiodurans*.

In summary, this thesis work presents a good fundament for further research on the *D. radiodurans* DNA repair systems. Further studies should address the identification of novel DNA repair components, the interplay between these factors as well as the regulation of the various DNA repair pathways. These results would possibly be transferable to several other prokaryotes and to some extend also to DNA repair mechanisms in eukaryotes and lead to a better understanding of DNA strand-break repair in general.

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M. Blasius, R. Buob, I. Shevelev, and U. Hübscher, DNA ligation and end-healing in the radioresistant bacterium *Deinococcus radiodurans*, *BMC Mol Biol*, 8:69, doi:10.1186/1471-2199-8-69

M. Blasius, I. Shevelev, E. Jolivet, S. Sommer, and U. Hübscher, DNA polymerase X from *Deinococcus radiodurans* possesses a structure-modulated 3’-5’ exonuclease activity involved in radioresistance, *Mol Microbiol*, 60, 165-176, 2006

M. Svetlova, L. Solovjeva, M. Blasius, I. Shevelev, U. Hübscher, P. Hanawalt and N. Tomilin, Differential incorporation of halogenated deoxyuridines during UV-induced DNA repair synthesis in human cells, *DNA Repair*, 4, 359-366, 2005

APPENDIX

A. Differential incorporation of halogenated deoxyuridines during UV-induced DNA repair synthesis in human cells

Reprinted from *DNA Repair* (2005), **4**, 359-366

My contribution to this paper was to test the *in vitro* utilisation of I-dUTP by DNA polymerases δ and ε.

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Differential incorporation of halogenated deoxyuridines during UV-induced DNA repair synthesis in human cells

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Abstract

Double labeling of interphase and metaphase chromosomes by 5-chlorodeoxyuridine (CldU) and 5-iododeoxyuridine (IdU) has been used in studies of the dynamics of DNA replication. Here, we have used this approach and confocal microscopy to analyze sites of DNA repair synthesis during nucleotide excision repair (NER) in quiescent human fibroblasts. Surprisingly, we have found that when both precursors are added at the same time to UV-irradiated cells they label different sites in the nucleus. In contrast, even very short periods of simultaneous IdU + CldU labeling of S-phase cells produced mostly overlapped IdU and CldU replication foci. The differential labeling of repair sites might be due to compartmentalization of I-dUTP and Cl-dUTP pools, or to differential utilization of these thymidine analogs by DNA polymerases δ and ε (Polδ and Polε). To explore the latter possibility we used purified mammalian polymerases to find that I-dUTP is efficiently utilized by both Polδ and Polε. However, we found that the UV-induced incorporation of IdU was more strongly stimulated by treatment of cells with hydroxyurea than was incorporation of CldU. This indicates that there may be distinct IdU and CldU-derived nucleotide pools differentially affected by inhibition of the ribonucleotide reductase pathway of dNTP synthesis and that is consistent with the view that differential incorporation of IdU and CldU during NER may be caused by compartmentalization of IdU- and CldU-derived nucleotide pools.

Keywords: Nucleotide excision repair; DNA replication; Human fibroblasts

1. Introduction

DNA replication foci, as discrete sites of incorporation of 5-bromodeoxyuridine (BrdU), have been detected in the mammalian cell nucleus by indirect immunofluorescence using anti-BrdU antibodies [1–3]. Some antibodies prepared against BrdU in DNA can also be used for analysis of the replicative incorporation of 5-iododeoxyuridine (IdU) and 5-chlorodeoxyridine (CldU) [4–11]. Detection of incorporated IdU has also been used for high-resolution analysis of DNA repair synthesis foci during nucleotide excision repair (NER) in human cells [12–13].

Double in vivo labeling of replication based upon the discrimination between different halogenated deoxyuridines by different anti-BrdU antibodies was originally proposed for cell kinetic studies [4] and then used in studies of the dynamics of DNA replication foci during S-phase [6,10], for analysis of the relationships between chromosome territories [14] and for discrimination (in the interphase nucleus or in metaphase chromosomes) of large chromatin domains that replicated early or late in S-phase [15–18]. Three DNA polymerises (Polα, Polδ and Polε) are known to be utilized in eukaryotic chromosomal DNA replication [19]. Recent studies

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have indicated that in mammalian cells Polε may be specifically involved in replication during late S-phase [20] when large blocks of heterochromatin are duplicated [3].

However, double labeling with halogenated deoxyuridines has not yet been applied to an analysis of repair synthesis during NER in human cells. Two major subpathways of NER are known: global genome repair (GGR) and transcription-coupled repair (TCR), which preferentially eliminates lesions from the transcribed DNA strand of expressed genes [21]. The final step in both NER subpathways is repair synthesis, which is performed by Polδ and Polε [22] to produce a patch of roughly 30 nucleotides [23], which is then ligated to contiguous DNA. At least some of the repair synthesis patches in TCR and GGR are clustered into discrete foci in the nucleus [12,13]. Here, we present results of our studies of repair synthesis in UV-irradiated human cells using double labeling with IdU and CldU. Initially, we had hoped to use sequential labeling with IdU and CldU to investigate the possible temporal heterogeneity of repaired chromosomal domains in NER. Surprisingly, we found differential repair synthesis labeling in nuclei and in chromosomes, even upon simultaneous addition of these precursors to UV-irradiated quiescent cells.

2. Materials and methods

2.1. Cell cultures and ultraviolet (UV) irradiation

Primary diploid human fibroblasts from human embryonic skin were cultured in minimal essential medium (MEM) supplemented with 2 mM glutamine, 100 μg/ml penicillin and streptomycin, and 10% fetal calf serum (FCS). For DNA repair experiments the cells were grown to confluence on glass microscope slides in Petri dishes and were incubated in MEM containing only 0.5% FCS during 1–2 days to attain the quiescent state. For labeling during replication, growing cells were used: one day before the experiment the medium containing 0.5% FCS was added. After washing with phosphate-buffered saline (PBS), cells were UV-irradiated with 30 J/m^2 from a germicidal lamp (254 nm). UV-dosimetry employed a commercial dosimeter equipped with the appropriate (254 nm) sensor.

2.2. Incorporation of thymidine analogs and fixation

For simultaneous DNA repair labeling after UV irradiation cells were incubated for 3 h or 20 h in MEM supplemented with 0.5% FCS, 10 μM 5-fluorodeoxyuridine (FdU), 10 μM 5-iododeoxyuridine and 10 μM 5-chlorodeoxyuridine (Sigma). For simultaneous DNA replication labeling in growing cells, 10 μM FdU, 10 μM IdU and 10 μM CldU were added for only 5 or 10 min in MEM containing 10% FCS. After washing two times with PBS, slides were fixed for 10 min in ice-cold 4% formaldehyde in PBS and kept in 70% ethanol at 4 °C.

2.3. IdU/CldU-staining procedure for detection of DNA replication and DNA repair double-labeled sites

Following overnight storage in 70% ethanol at 4 °C, slides were rinsed with PBS and incubated for 40 min in 0.5% Triton X-100 solution in PBS with gentle shaking at room temperature; then slides were rinsed with PBS. For denaturation of DNA in the nuclei, the slides were placed in 4N HCl for 30 min at room temperature, then washed three times with PBS and incubated in 1% "blocking reagent" (Roche)/ 5% BSA in PBS with 0.02% Tween 20. Antibodies and other detection reagents were diluted in PBS supplemented with 0.5% "blocking reagent" and 0.02% Tween 20, and incubations were carried out at 37 °C in a humid chamber. For double IdU/CldU staining the modified protocol described in reference [5] was used. The primary incubation was carried out with the mixture of monoclonal rat anti-bromodeoxyuridine (BrdU) antibodies (Ab) (SeraLab MAS-250, clone BU1/75, 1:200) (that also reacts with CldU) and mouse anti-BrdU (Becton Dickinson, clone B44, 1:5) Ab (that also reacts with IdU and CldU) for 1 h. Then, slides were incubated for 6 min in Tris buffer containing 0.5 M NaCl, 37 mM Tris–HCl pH 8, 0.5% Tween 20 at room temperature with shaking. During this procedure, the molecules of mouse anti-BrdU Ab that had less affinity to CldU than to IdU, were washed from the CldU attachment sites. Slides were washed three times with PBS supplemented with 0.2% Tween 20 and rinsed with 0.5% solution of "blocking reagent" as indicated above. Between the following incubations with detection reagents, slides were washed in the same way.

Slides were incubated for the second time with rat anti-BrdU Ab (Sera Lab, 1:200) to let that antibody occupy the CldU-attachment sites that had been released from mouse anti-BrdU Ab (Becton Dickinson) during incubation in Tris buffer. They were then washed, incubated for 20 min with 50% normal sheep serum (Jackson Laboratories) in PBS, rinsed with 0.5% "blocking reagent" solution and incubated for 40 min with the mixture of biotinylated sheep anti-mouse IgG Ab (Sigma, 1:100) and rabbit anti-rat IgG Ab (DAKO, 1:100), then washed. Slides were incubated with 50% normal goat serum (Jackson Laboratories) in PBS, rinsed with 0.5% "blocking reagent" solution, incubated for 40 min with the mixture of FITC-conjugated goat anti-rabbit IgG Ab (Roche, 1:200) and Texas Red-conjugated avidin (Oncor, 1:50), and washed. For detection of DNA repair sites, the red IdU signal was additionally amplified with biotinylated goat anti-avidin Ab (Vector, 1:100, 40 min) and Texas Red-conjugated avidin (Oncor, 1:50, 40 min), and washed. Slides were mounted in CITIFLUOR antifading solution (glycerol/PBS solution, UKS Chemical Laboratories, UK).

2.4. Microscopy and image processing

Confocal images were acquired using a Zeiss LSM5 Pascal system (Figs. 1 and 3) or an Olympus epifluorescence microscope, and recorded using Vario Cam CCD-camera

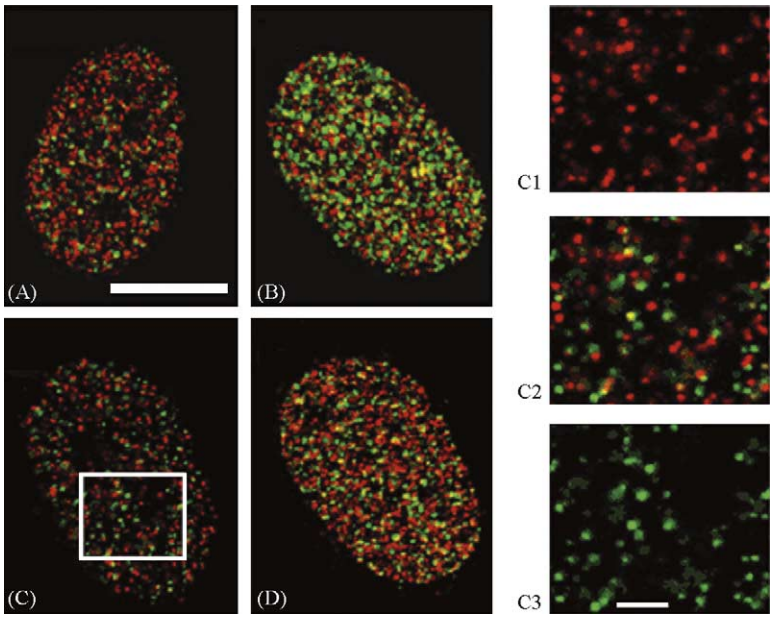

Fig. 1. Visualization of CldU (green) and IdU (red) incorporated simultaneously during DNA repair synthesis in UV-irradiated quiescent human fibroblasts. (A and B) show two cells incubated with IdU and CldU (10 μM each) for 3 h (A) or 20 h (B) after irradiation with UV dose 30 J/m^2; (C and D) shows two cells incubated with IdU + CldU for 20 h after 5 J/m^2 (C) or 20 J/m^2 (D). (C1–C3) show a segment of nucleus (C, boxed) at approximately two-fold higher magnification. Confocal images obtained using a Zeiss LSM5 Pascal system are shown. Bar in (A) is 10 μm and in (C3) is 2 μm.

(Germany) with the help of the KS-100 program. Images were processed and analyzed with the use of Adobe Photoshop version 6.

2.5. Analysis of I-dUTP utilization by purified mammalian Polδ and Polε

DNA polymerase δ (Polδ) was isolated from calf thymus and DNA polymerase ε (Polε) was isolated from HeLa cells as described [24]. In the filter assay, 25 μl of reaction mixture was set up containing the following components: 50 mM HEPES-KOH (pH 7.5), 15 mM $MgCl_2$, 100 mM KCl, 10 mM dithiothreitol, 0.03% Triton X-100, 20% glycerol, 0.2 mg/ml BSA, 1 μl of Polε or Polδ, 2 μg gapped calf thymus DNA, 2 μM (1 μCi) ^{3}H-dCTP (Amersham) and 25 μM of each dNTP. All reactions were incubated for 30 min at 37 °C. For comparing the incorporation of I-dUTP and dTTP, the standard assay was used and only the dTTP/I-dUTP concentration was varied. I-dUTP (5-iodo-2′-deoxyuridine triphosphate) was obtained from Trilink BioTechnologies company. The reactions were stopped by adding 800 μl of 10% trichloroactetic acid and placing the reaction mixture directly on ice. The DNA was allowed to precipitate for at least 10 min. The precipitates were collected on Millipore HA filters, washed twice with HCl/sodium diphosphate, once with ethanol and then dried for several minutes under a heat lamp. The filter-bound radioactivity was determined by adding 2 ml of scintillation fluid and counting in a liquid scintillation counter. 375 cpm corresponded to 1 pmol of incorporated nucleotides. The Polδ primer extension assay with poly A (60-mer) template and ^{32}P labeled (dT) 15 primer was carried out as described in reference [24]; final concentrations of I-dUTP and dTTP for this assay were 25 μM.

3. Results and discussion

3.1. Differential CldU/IdU labeling of nuclei in human fibroblasts during UV-induced repair synthesis

Focal sites of incorporation of IdU in UV-irradiated NER-proficient cells can be detected after very short times (<10 min) of incubation of the cells with IdU, using the Tyramide system of signal amplification [13] but this system does not work for double-labeling with IdU and CldU because of problems with background. Therefore, we used standard immunofluorescence and rather long incubation times (3 and 20 h) of the quiescent UV-irradiated cells with these precursors, which were added to cells either separately or simultaneously at 10 μM. Since completely colocalised IdU and CldU foci have usually been observed upon simultaneous addition of these precursors to S-phase cells [10,15,18], we expected to also find colocalised repair synthesis foci in UV-irradiated cells upon simultaneous addition of IdU and CldU. Surprisingly, extensive differential labeling of nuclei in UV-irradiated cells was reproducibly observed after simultaneous incubation with IdU and CldU for 3 h following 30 J/m^2 (Fig. 1A) or for 20 h after 5, 20 or 30 J/m^2 (Fig. 1C, D and B, respectively). Fig. 1C1–C3 shows a segment of one nucleus (Fig. 1C, boxed) at a higher magnification, illustrating very little overlap of the green (CldU) and red (IdU) foci.

Table 1
Quantitation of IdU and CldU foci in UV-irradiated cells

UV-dose ($J\,m^{-2}$)	Incubation time with IdU + CldU (h)	Number of foci per nucleus[a]		Mean overall luminosity (red + green)	Mean red signal	Mean green signal
		Red	Green			
30	3	1038 ± 118	434 ± 57	12.9 ± 2.8	26.6 ± 5.2	8.3 ± 3.4
30	20	1338 ± 92	810 ± 94	34.2 ± 2.9	45.2 ± 5.5	34.9 ± 4.8
Ratio 20 h/3 h		1.29	1.87	2.65	1.70	4.20
5	20	776 ± 54	634 ± 40	12.1 ± 1.6	18.8 ± 4.3	11.0 ± 1.0
20	20	1383 ± 69	1175 ± 159	27.6 ± 1.0	42.2 ± 3.5	25.3 ± 3.4
Ratio 20 $J\,m^{-2}$ to 5 $J\,m^{-2}$		1.78	1.85	2.28	2.24	2.27

[a] In the absence of UV irradiation the number of green and red foci detected 3 h after incubation of cells with halogenated deoxyuridines was <30 per nucleus.

Confocal images like those shown in Fig. 1 facilitate comparative analysis of the overall intensity of fluorescence (IF), registered using the same amplification gain and pinhole, as well as counting the number of repair foci after different times of labeling and various UV doses. We found that after 20 h the number of both red and green foci is increased compared to that after 3 h of labeling; but the overall IF increases at higher rate than does the number of foci, indicating that there is a net increase in the mean IF per single focus (Table 1). Therefore, new foci of repair synthesis appear after 3 h of initial repair while repair is still going on in foci already visible during that first 3 h of labeling. The new repair foci seen after 20 h of labeling may have arisen at sites which had been primed for repair during the first 3 h, but which were not yet visible because of a very weak IF signal, below the detection threshold.

The number of foci after 20 h of labeling also increases as a function of UV dose (Fig. 1C and D) but this increase is not directly proportional to the UV dose (Table 1). There is no further increase in the number of foci after 20 h incubation following UV doses higher than 20 J/m^2 (Table 1), and after 20 J/m^2 there is only a moderate increase of the IF per focus compared to that after 5 J/m^2. Although colocalizing green and red (seen as yellow) foci are evident after 20 J/m^2 rather than after 5 J/m^2 (Fig. 1C and D), this colocalization increases in parallel with the overall density of foci (Fig. 2A), consistent with the view that it reflects random overlap of these foci rather than bonafide colocalization.

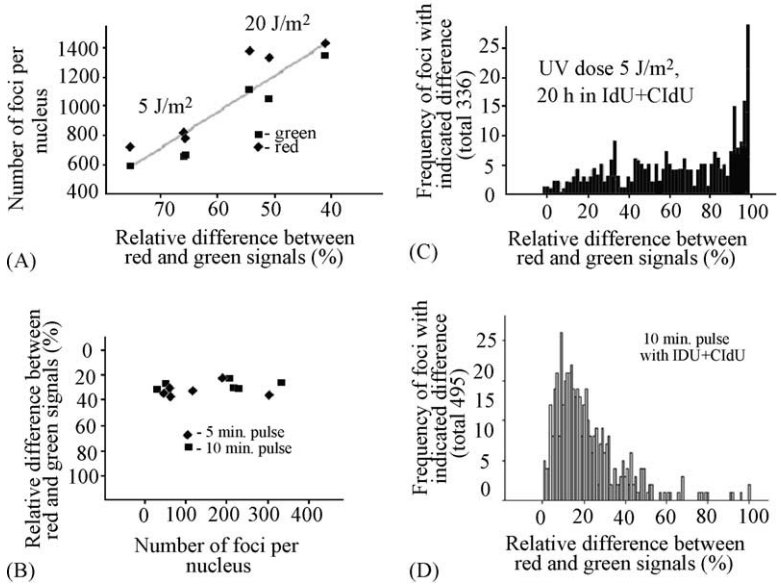

Fig. 2. Results of analysis of confocal images of simultaneous incorporation of IdU and CldU during UV-induced repair synthesis in quiescent cells (A and C) or normal replication in undamaged S-phase cells (B and D). Differences and sums of red and green signals on confocal RGB images collected at the same amplification gains and pinholes were calculated (after elimination of the blue color) using Photoshop's "Difference" and "Add" functions. Relative differences were calculated from "Difference" and "Add" grey images by dividing corresponding values obtained over the same nuclear areas using Photoshop "Histogram" function. In (A and B) large areas of nuclei with hundreds of foci were compared and in (C and D) individual repair (C) or replication foci (D) were analyzed and compared.

3.2. IdU/CldU labeling of unirradiated S-phase cells

In contrast to the results for UV-irradiated non-S-phase cells, even very short labeling (5 and 10 min) of unirradiated S-phase cells with both IdU and CldU under similar experimental conditions for detection exhibited strongly colocalized IdU and CldU foci (Fig. 3A and B), and in this case the overlap of red and green signals was clearly independent of the density of the foci (Fig. 2B). It is also apparent in Fig. 3 (arrows) that many very small and weakly-labeled foci also show colocalization of green and red signals suggesting that: (1) this coincidence is not random and is a consequence of the simultaneous incorporation of IdU and CldU into the same nuclear domains, and (2) the method of double-labeling used in this study allows detection of even weak simultaneous incorporation of IdU and CldU into the same subchromosomal domains. Therefore, it is unlikely that the strong differential labeling of small repair foci is due to random variations in the binding of the antibodies. Such variations could probably explain a rather wide distribution in the relative intensities of red and green signals in individual repair (Fig. 2C) or replication foci (Fig. 2D). However, the histogram for repair synthesis foci is clearly shifted to the right (i.e., greater difference) compared to that for the replication foci, which peaks at less than a 30% difference between red and green signals. Differential labeling of nuclei with IdU and CldU during NER might also be due to differential accessibility of antibodies to IdU- and CldU-containing epitopes in repaired subchromosomal genome segments.

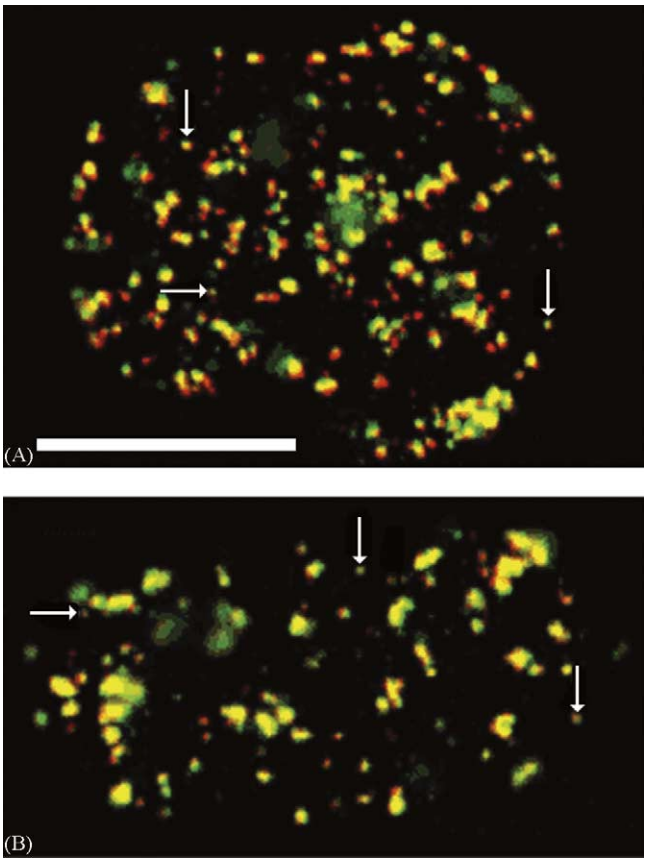

Fig. 3. Visualization of replication foci in undamaged S-phase cells after simultaneous incubation with IdU and CldU for 5 min (A) and 10 min (B). Arrows show small replication foci with colocalized IdU (red) and CldU (green) signals. Bar is 10 μm.

In this study, in order to examine IdU/CldU repair epitopes we treated the formaldehyde-fixed cells for 30 min with 4N HCl, which allows reliable detection of all IdU or CldU incorporated during replication [5,10]. However, this method for detection of IdU/CldU in repair sites had not been validated in earlier work. Therefore, we measured the repair synthesis signal generated in IdU/CldU-labeled cells after different times of incubation of the fixed cells in 4N HCl or using alternative method of in situ DNA denaturation (40 min at 70 °C in 0.1× PBS–90% formamide). We found no further increase of IdU or CldU repair synthesis signal after incubation for longer than 30-min (up to 80 min) of fixed cells in 4N HCl; and weaker repair synthesis signals were obtained using the alternative method of DNA denaturation (data not shown), suggesting that the differential labeling of cells during NER with IdU and CldU is unlikely to be due to differential in situ accessibility of these molecules to the respective antibodies. Taken together, our results indicate that differential IdU/CldU labeling of the nuclear DNA during UV-induced repair synthesis (Fig. 1) is a consequence of variation in the incorporation of these halogenated deoxyuridines into different nuclear compartments.

3.3. Incorporation of I-dUTP in vitro by purified mammalian DNA Polδ and Polε

Since both DNA polymerases δ and ε (Polδ and Polε) are known to be involved in repair synthesis during NER [22], differential labeling of repair sites by IdU and CldU might be a consequence of differential utilization of the respective triphosphates by these polymerases, which might be partitioned into different nuclear compartments [19]. Some prokaryotic DNA polymerases have been shown to discriminate kinetically between thymidine analogs in a competitive assay [25]. Here, we studied incorporation of I-dUTP by purified Polδ and Polε in a primer extension assay with a poly dA template (Fig. 4A) and using a filter assay with gapped DNA as template (Fig. 4B). Extension of ^{32}P-labeled oligo dT primer by Polδ was equally efficient in the presence of PCNA and 25 μM I-dUTP or 25 μM dTTP (Fig. 4A). The filter assay (in which incorporation of ^{3}H-labeled dCTP into acid-insoluble material was analyzed) was performed at very low (1 nM) concentrations of I-dUTP and TTP and it revealed that both Polδ and Polε can efficiently incorporate ^{3}H-dCTP into DNA in the presence of I-dUTP (Fig. 4B). Significant incorporation of ^{3}H-dCTP by Polε was observed in the absence of dTTP or I-dUTP (Fig. 4B, bottom plot) but it was lower than that observed in the presence of 1 nM dTTP or I-dUTP.

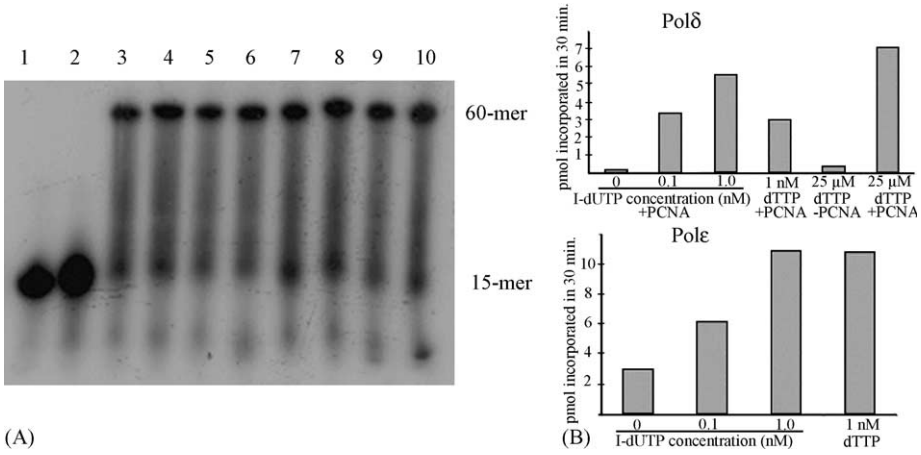

Fig. 4. Utilization of I-dUTP in vitro by purified DNA polymerases δ and ε. (A) Primer extension assay on poly dA template with DNA polymerase δ, radioautograph of reaction products separated in an agarose gel is shown; (B) filter assay with DNA polymerase δ (top plot) and DNA polymerase ε (bottom plot). Experimental details are described in Section 2 and in the main text. Lane 1 contained only ^{32}P-labeled primer (15-mer), lane 2 contained dTTP and Polδ without PCNA, incubation was 30 min, lanes 3–12 contained both PCNA and Polδ, dTTP was present in lanes 3–6 and I-dUTP was present in lanes 7–10. Incubation time was 5 min in lanes 3 and 7, 10 min in lanes 4 and 8, 20 min in lanes 5 and 9, and 30 min in lanes 6 and 10.

It may be also noted that at a concentration of 1 nM, I-dUTP was utilized by Polδ more efficiently than dTTP (Fig. 4B, top plot) suggesting that this polymerase may be able to perform kinetic discrimination between thymidine analogs. However, this possibility should be confirmed using a competitive assay. Taken together, our results indicate that both Polδ and Polε can efficiently utilize I-dUTP during DNA synthesis in vitro and that it is unlikely that selective IdU labeling of repair domains in UV-irradiated quiescent cells in vivo is due to the selective incorporation of I-dUTP by Polδ or Polε associated with different domains.

3.4. Treatment of cells with hydroxyurea stimulates in vivo incorporation of IdU and CldU

Many studies have identified two functionally distinct metabolic pathways that supply the cell with deoxynucleotides for DNA replication and DNA repair [26–32]. The de novo pathway, which depends upon ribonucleotide reductase (RNR)-dependent conversion of ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs) is the major source of DNA precursors during DNA replication in proliferating cells [26,27]. The salvage pathway, initiated by deoxycytidine kinase, makes little contribution to DNA replication but it accounts for the majority of dCMP incorporation into DNA during repair synthesis [27]. However, the relative contribution to replication from the salvage pathway-produced dTMP may be higher than that of dCMP. Other studies indicate that the two RNR subpathways can also play a crucial role in supplying dNTPs for DNA repair [28–32]. In one of the subpathways the RNR subunit, RRM2, is not induced by DNA damage and is expressed constitutively [28] and in the second RNR subpathway another subunit, P53R2, is induced in a p53-dependent fashion [28–30]. We examined whether UV-induced incorporation of IdU and CldU in quiescent cells is affected by treatment of the cells with HU, which inhibits activities of both the inducible (P53R2) and the constitutive (RRM2) subunits of RNR [32]. It is evident from Fig. 5 that HU stimulates UV-induced incorporation of both IdU and CldU, indicating that an inhibition of the de novo RNR-dependent pathway of dNTP synthesis increases the contribution from the salvage pathway initiated by thymidine kinase. This suggests that, in the absence of HU, both pathways compete to supply pyrimidine nucleotides for repair synthesis in quiescent human fibroblasts, and therefore, the analysis of NER by incorporation of thymidine analogs may underestimate the actual rate of DNA repair synthesis. In UV-irradiated quiescent human fibroblasts, IdU incorporation accounts for only about 20% of the actual repair synthesis observed in the presence of HU

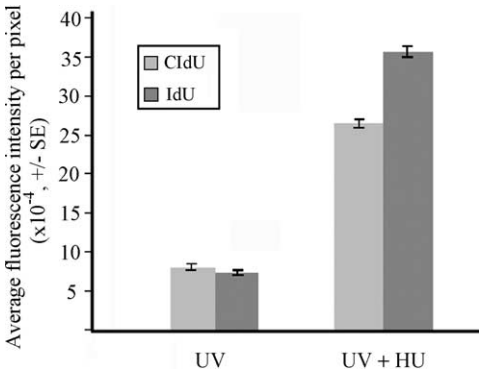

Fig. 5. Differential stimulation of UV-induced incorporation of IdU and CldU by hydroxyurea (HU). UV dose was 30 J/m^2, and HU (3 mM) was added 30 min before irradiation and then present during 2 h simultaneous labeling with IdU and CldU (10 μM each). Hundred cells were analyzed in each variant and background fluorescence observed in unirradiated control is subtracted.

(Fig. 5), and therefore, about 80% of the repair synthesis in these cells (dependent upon deoxynucleotides from the RNR pathway) remains unaccounted for. Commonly used ^{3}H-thymidine incorporation assays (e.g., for unscheduled DNA synthesis) can also result in an underestimation of the actual amount of repair synthesis, which can be diminished by the use of hydroxyurea. It is likely that the stimulation of IdU and CldU incorporation occurs because of the HU-mediated inhibition of the P53R2 subunit of RNR, although the involvement of the RRM2 subunit, which is more sensitive to HU inhibition than P53R2 [32], cannot be excluded.

Interestingly, the stimulation of IdU incorporation by hydroxyurea had been documented earlier in experiments with exponentially growing cells from a bladder cancer cell line 647 V, that had not received DNA damaging treatment [34]. This stimulation was due to a two-fold increase in the IdU pool within two hours after HU addition [34] and was probably dependent upon the inhibition of the constitutive RNR subunit RRM2. It seems therefore, that the thymidine kinase initiated salvage pathway competes with the RNR pathway in supplying nucleotides not only for repair synthesis, but also for DNA replication.

We have found that the extent of the HU-mediated stimulation of repair synthesis in UV-irradiated quiescent human fibroblasts is significantly higher for IdU than for CldU (Fig. 5), indicating differences in the processing of IdU and CldU by the salvage pathway. It is known, for example, that CldU (but not IdU) can inhibit thymidylate synthase [33], thereby reducing the generation of endogeneous dTMP, which would otherwise interfere with incorporation of halogenated deoxyuridines. Differential stimulation of IdU and CldU incorporation by HU suggests that functionally biased CldU- and IdU-derived nucleotide pools for repair synthesis may exist in the nucleus. Our observation of differential labeling of repair synthesis sites (Fig. 1) is consistent with the view that the CldU- and IdU-derived nucleotide pools in nuclei are not only functionally biased but also structurally compartmentalized, which may depend upon an uneven cellular distribution of some enzymes in the salvage pathway in quiescent cells, discriminating IdU and CldU. In S-phase cells, these enzymes may be uniformly distributed and so no differential labeling of replication sites is observed (Fig. 3). Further studies of the distribution of the enzymes of the salvage and RNR pathways may be helpful to establish the basis for compartmentalization of IdU- and CldU-derived nucleotide pools used in UV-induced DNA repair synthesis in quiescent human fibroblasts.

Stimulation of the incorporation of IdU and CldU by hydroxyurea may have implications for treatment of slowly proliferating tumors, since this incorporation can increase tumor radiosensitivity [34]. DNA repair-dependent incorporation of IdU and CldU into DNA can be stimulated by the introduction of additional DNA damage into tumor cells by chemical genotoxins such as 4-nitroquinoline-1-oxide and methyl methanesulfonate [35].

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